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MOLECULAR BIOLOGY OF THE CELL

SECOND EDITION

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson

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phate), and is inhibited by ATP, which is one of its substrates. As a result, this enzyme can turn itself on, being subject to a complex form of positive feedback control. Under certain circumstances such feedback control gives rise to striking oscillations in the activity of the enzyme, causing corresponding oscillations in the concentrations of various glycolytic intermediates (Figure 2–39). Although the physiological significance of these particular oscillations is not known, they illustrate how a biological oscillator can be produced by a few enzymes. In principle, such oscillations could provide an internal clock, enabling a cell to "measure time" and, for example, to perform certain functions at fixed intervals.

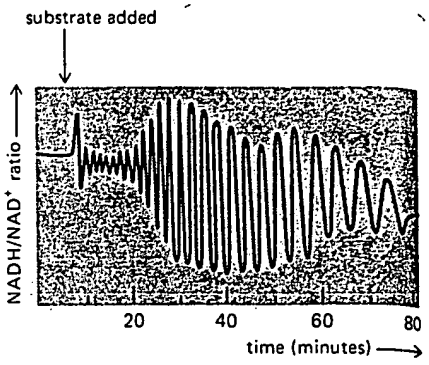


Figure 2–39 The abrupt addition of glucose to an extract containing the enzymes and cofactors required for glycolysis can produce large cyclic fluctuations in the levels of intermediates such as NADH. These metabolic oscillations arise, in part, from the positive feedback control of the glycolytic enzyme phosphofructokinase.

Enzymes Can Be Switched On and Off by Covalent Modification[22]

The types of feedback control just described permit the rates of reaction sequences to be continuously and automatically regulated in response to second-by-second fluctuations in metabolism. Cells have different devices for regulating enzymes when longer-lasting changes in activity, occurring over minutes or hours, are required. These involve reversible covalent modification of enzymes, which is often, but not always, accomplished by the addition of a phosphate group to a specific serine, threonine, or tyrosine residue in the enzyme. The phosphate comes from ATP, and its transfer is catalyzed by a family of enzymes known as *protein kinases*.

We shall describe in the following chapter how phosphorylation can alter the shape of an enzyme in such a way as to increase or inhibit its activity. The subsequent removal of the phosphate group, which reverses the effect of the phosphorylation, is achieved by a second type of enzyme, called a *phosphoprotein phosphatase*. Covalent modification of enzymes adds another dimension to metabolic control because it allows specific reaction pathways to be regulated by signals (such as hormones) that are unrelated to the metabolic intermediates themselves.

Reactions Are Compartmentalized Both Within Cells and Within Organisms[23]

Not all of a cell's metabolic reactions occur within the same subcellular compartment. Because different enzymes are found in different parts of the cell, the flow of chemical components is channeled physically as well as chemically.

The simplest form of such spatial segregation occurs when two enzymes that catalyze sequential reactions form an enzyme complex, and the product of the first enzyme does not have to diffuse through the cytoplasm to encounter the second enzyme. The second reaction begins as soon as the first is over. Some large enzyme aggregates carry out whole series of reactions without losing contact with the substrate. For example, the conversion of pyruvate to acetyl CoA proceeds in three chemical steps, all of which take place on the same large enzyme complex (Figure 2–40), and in fatty acid synthesis an even longer sequence of reactions is catalyzed by a single enzyme assembly. Not surprisingly, some of the largest en-

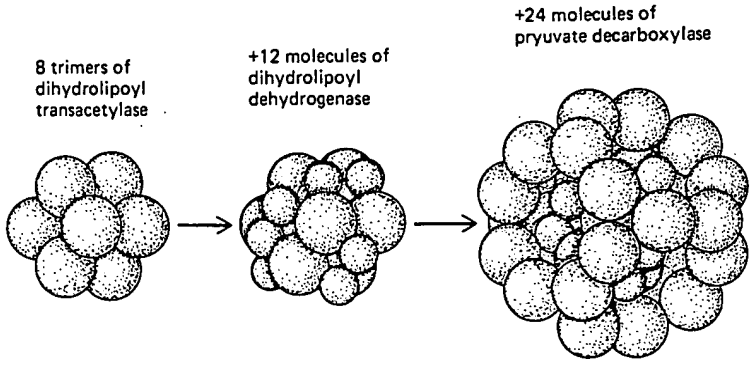
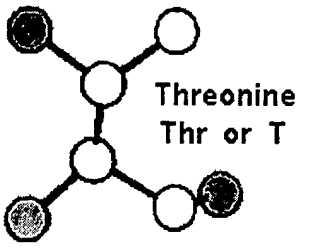
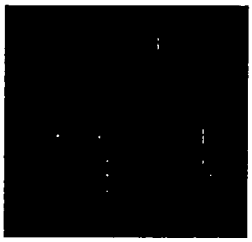


Figure 2–40 The structure of pyruvate dehydrogenase—an example of a large multienzyme complex in which reaction intermediates are passed directly from one enzyme to another. This enzyme complex catalyzes the conversion of pyruvate to acetyl CoA.

Threonine





Standard codons for T : ACA ACC ACG ACT

Substitution preferences:

All protein types:

Favoured	Ser (1)
Neutral	Ala (0) Asn (0) Val (0)
Disfavoured	Asp (-1) Glu (-1) Ile (-1) Lys (-1) Met (-1) Leu (-1) Pro (-1) Gln (-1) Arg (-1) Cys (-1) Phe (-2) His (-2) Trp (-2) Tyr (-2) Gly (-2)

Intracellular proteins:

Favoured	
Neutral	Cys (0) Asp (0) Glu (0) Lys (0) Met (0) His (0) Ile (0) Asn (0) Pro (0) Gln (0) Arg (0) Ser (0) Ala (0) Val (0)
Disfavoured	Leu (-1) Phe (-1) Tyr (-1) Gly (-1) Trp (-2)

Extracellular proteins:

Favoured	Ser (1)
Neutral	Ala (0) Val (0) Asp (0) Glu (0) Gly (0) His (0) Ile (0) Lys (0) Met (0) Leu (0) Asn (0) Pro (0) Gln (0) Arg (0)
Disfavoured	Trp (-1) Phe (-1) Tyr (-1) Cys (-5)

Membrane proteins:

Favoured	Ser (2) Asn (1) Ala (1)
Neutral	Gly (0) Ile (0) Met (0) Cys (0) Asp (0) Val (0)
Disfavoured	Pro (-1) Glu (-1) Arg (-1) Leu (-1) His (-2) Phe (-2) Lys (-2) Gln (-2) Tyr (-3) Trp (-4)

Substitutions: As Threonine is generally considered a slightly polar *polar*, amino acid, though it is fairly neutral with regard to mutations, though generally it subsitutes with other *polar* or *small*

amino acids, in particular Serine which differs only in that it has a hydrogen in place of the methyl group found in Threonine.

Role in structure: Being a fairly indifferent amino acid, Threonine can reside both within the interior of a protein, or on the protein surface.

Threonine has an additional property that is frequently overlooked. Like Valine, and Isoleucine it is *C-beta branched*. Whereas most amino acids contain only one non-hydrogen substituent attached to their C-beta carbon, these three amino acids contain two. This means that there is a lot more bulkiness near to the protein backbone, and thus means that these amino acids are more restricted in the conformations the main-chain can adopt. Perhaps the most pronounced effect of this is that it is more difficult for these amino acids to adopt an alpha-helical conformation, though it is easy and even preferred for them to lie within beta-sheets.

Role in function: Threonines are quite common in protein functional centres. The hydroxyl group is fairly reactive, being able to form hydrogen bonds with a variety of polar substrates.

A common role for Threonines (and Serines and Tyrosines) within intracellular proteins is phosphorylation. Protein kinases frequently attach phosphates to Threonines in order to fascilitate the signal transduction process.

Note that in this context, Threonine can often be replaced by Serine, but is unlikely to be replaced by Tyrosine, as the enzymes that catalyse the reactions (i.e. the protein kinases) are highly specific (i.e. Tyrosine kinases generally do not work on Serines/Threonines and *vice versa*).

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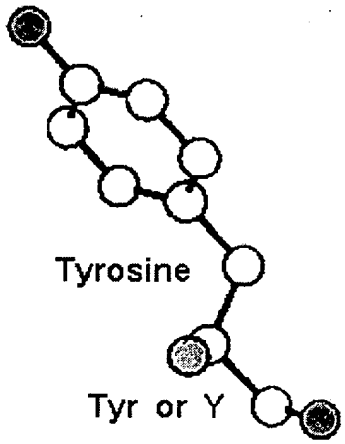
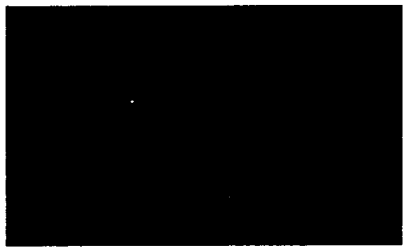
Tyrosine





Standard codons for Y : TAC TAT

Substitution preferences:

All protein types:

Favoured	Phe (3) Trp (2) His (2)
Disfavoured	Met (-1) Leu (-1) Gln (-1) Val (-1) Ile (-1) Ala (-2) Lys (-2) Cys (-2) Glu (-2) Ser (-2) Arg (-2) Thr (-2) Asn (-2) Asp (-3) Pro (-3) Gly (-3)

Intracellular proteins:

Favoured	Trp (2) Phe (2) His (1)
Neutral	Leu (0) Cys (0) Val (0) Ile (0) Met (0)
Disfavoured	Ala (-1) Asn (-1) Glu (-1) Pro (-1) Gln (-1) Arg (-1) Ser (-1) Thr (-1) Lys (-1) Asp (-2) Gly (-2)

Extracellular proteins:

Favoured	Phe (2) Trp (1)
Neutral	Val (0) Ile (0) His (0) Arg (0)
Disfavoured	Ala (-1) Glu (-1) Lys (-1) Met (-1) Leu (-1) Asn (-1) Pro (-1) Gln (-1) Ser (-1) Thr (-1) Gly (-2) Asp (-2) Cys (-4)

Membrane proteins:

Favoured	His (6) Cys (3) Phe (2) Lys (1)
Neutral	Gln (0) Ser (0)
Disfavoured	Arg (-1) Asn (-1) Asp (-2) Leu (-2) Thr (-3) Trp (-3) Ala (-3) Met (-3) Ile (-4) Val (-4) Pro (-5) Glu (-5) Gly (-5)

Substitutions: As Tyrosine is an *aromatic*, partially *hydrophobic*, amino acid, it prefers substitution with other amino acids of the same type (see above). It particularly prefers to exchange with Phenylalanine, which differs only in that it lacks the hydroxyl group in the *ortho* position on the benzene ring.

Role in structure: Being partially hydrophobic, Tyrosine prefers to be buried in protein hydrophobic cores. The aromatic side chain can also mean that Tyrosine is involved in *stacking* interactions with other *aromatic* side-chains.

Role in function: Unlike the very similar Phenylalanine, Tyrosine contains a reactive hydroxyl group, thus making it much more likely to be involved in interactions with non protein atoms.

Like other *aromatic* amino acids, Tyrosine can be involved in interactions with non-protein ligands that themselves contain aromatic groups via *stacking* interactions.

Like other *aromatic* amino acids, Tryptophan can be involved in interactions with non-protein ligands that themselves contain aromatic groups via *stacking* interactions. Tryptophan and other *aromatic* amino acids can be involved in binding to poly-proline containing peptides, for example, in SH3 or WW domains.

A common role for Tyrosines (and Serines and Threonines) within intracellular proteins is phosphorylation. Protein kinases frequently attach phosphates to Tyrosines in order to fascilitate the signal transduction process. Note that in this context, Tyrosine will rarely substitute for Serine or Threonine, since the enzymes that catalyse the reactions (i.e. the protein kinases) are highly specific (i.e. Tyrosine kinases generally do not work on Serines/Threonines and *vice versa*).

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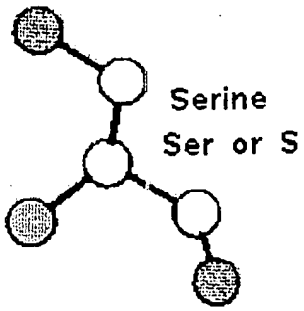
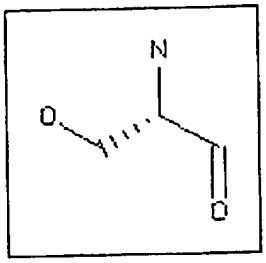
Serine





Standard codons for S : AGC AGT TCA TCC TCG TCT

Substitution preferences:

All protein types: not in TM core because also phosphorylatable

Favoured	Ala (1) Asn (1) Thr (1)
Neutral	Glu (0) Lys (0) Gly (0) Gln (0) Asp (0)
Disfavoured	Arg (-1) Met (-1) His (-1) Pro (-1) Cys (-1) Leu (-2) Val (-2) Phe (-2) Tyr (-2) Ile (-2) Trp (-3)

Intracellular proteins:

Favoured	
Neutral	Cys (0) Asp (0) Glu (0) Lys (0) Gly (0) His (0) Asn (0) Pro (0) Gln (0) Arg (0) Ala (0) Thr (0)
Disfavoured	Val (-1) Tyr (-1) Met (-1) Phe (-2) Trp (-2) Ile (-2) Leu (-2)

Extracellular proteins:

Favoured	Thr (1)
Neutral	Pro (0) Asp (0) Glu (0) Asn (0) Gly (0) His (0) Lys (0) Arg (0) Ala (0) Gln (0)
Disfavoured	Ile (-1) Met (-1) Leu (-1) Val (-1) Trp (-1) Tyr (-1) Phe (-2) Cys (-5)

Membrane proteins:

Favoured	Asn (2) Thr (2) Ala (2) Cys (1) Gly (1)
Neutral	Glu (0) Tyr (0) Asp (0)
Disfavoured	Pro (-1) Gln (-1) Phe (-1) Lys (-1) Arg (-1) Val (-1) Ile (-1) His (-2) Leu (-2) Met (-2) Trp (-3)

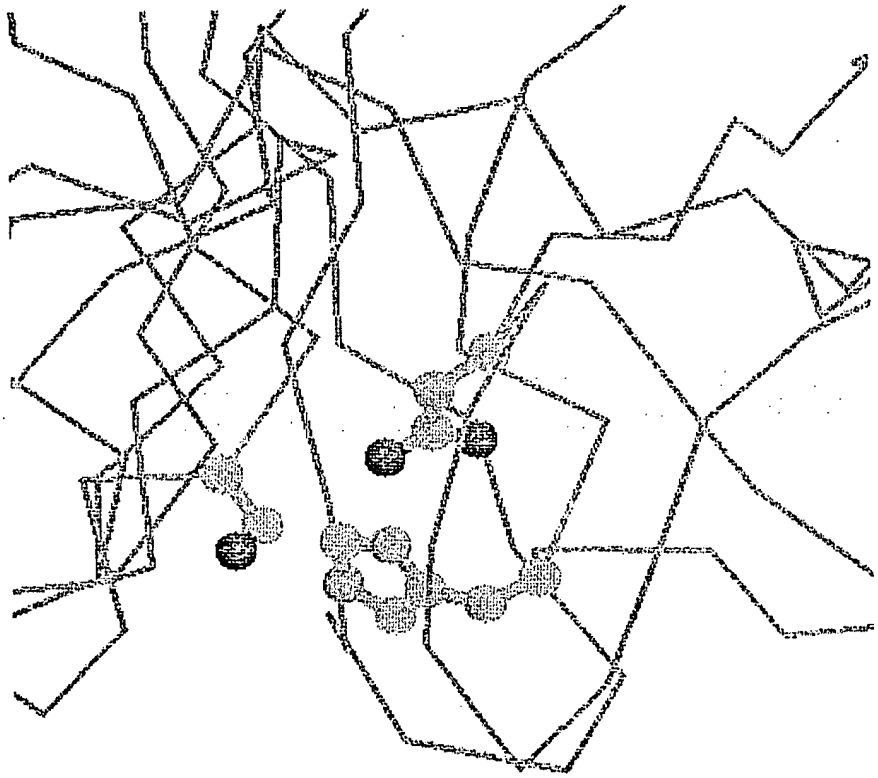
Substitutions: As Serine is generally considered a slightly polar *polar*, amino acid, though it is fairly neutral with regard to mutations, though generally it subsitutes with other *polar* or *small* amino acids,

in particular Threonine which differs only in that it has a methyl group in place of a hydrogen group found in Serine.

Role in structure: Being a fairly indifferent amino acid, Serine can reside both within the interior of a protein, or on the protein surface. Its small size means that it is relatively common within tight turns on the protein surface, where it is possible for the Serine side-chain hydroxyl oxygen to form a hydrogen bond with the protein backbone, effectively mimicking Proline.

Role in function: Serines are quite common in protein functional centres. The hydroxyl group is fairly reactive, being able to form hydrogen bonds with a variety of polar substrates.

Perhaps the best known role for Serine in protein active sites is found in the classical Asp-His-Ser *catalytic triad* found in many hydrolases (e.g. proteases, lipases, etc.). Here, a Serine, aided by a Histidine and an Aspartate acts as a nucleophile to hydrolyse (effectively cut) other molecules.



This three-dimensional `motif' is found in many non-homologous (i.e. un-related) proteins, and is a classic example of molecular convergent evolution. Note that in this context, it is rare for Serine to exchange with Threonine, but in some cases, the reactive serine can be replaced by Cysteine, which can perform a similar role.

A common role for Serines (and Threonines and Tyrosines) within intracellular proteins is phosphorylation. Protein kinases frequently attach phosphates to Serines in order to fascilitate the signal transduction process. Note that in this context, Serine can often be replaced by Threonine, but is unlikely to be replaced by Tyrosine, as the enzymes that catalyse the reactions (i.e. the protein kinases) are highly specific (i.e. Tyrosine kinases generally do not work on Serines/Threonines and *vice versa*).

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Phosphorylation of the Human Vitamin D Receptor by Protein Kinase C

BIOCHEMICAL AND FUNCTIONAL EVALUATION OF THE SERINE 51 RECOGNITION SITE*

(Received for publication, March 10, 1993, and in revised form, April 5, 1993)

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We have reported previously that the human vitamin D receptor (hVDR) is selectively phosphorylated by protein kinase C-β (PKC-β), *in vitro*, on a serine residue in the sequence RRS$_{51}$MKRK, which is located between the two zinc fingers of hVDR and is potentially important to its transacting function (Hsieh, J.-C., Jurutka, P. W., Galligan, M. A., Terpening, C. M., Haussler, C. A., Samuels, D. S., Shimizu, Y., Shimizu, N., and Haussler, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 9315–9319). In the present experiments we evaluated this phosphorylation event using a series of hVDR mutants in which serine 51 or its flanking residues were modified. Alteration of serine 51 to a nonphosphorylatable residue resulted in an approximately 60% reduction in basal hVDR phosphorylation in intact cells but did not diminish 1,25-dihydroxyvitamin D$_3$-stimulated phosphorylation. Such mutations also abolished subsequent phosphorylation of immunoprecipitated hVDR by purified PKC-β, *in vitro*, as did replacement of basic residues on either side of serine 51. Mutation of serine 51 to glycine (S51G) or to aspartic acid (S51D), as well as altering the basic residues flanking serine 51, abolished the interaction of hVDR with the vitamin D-responsive element (VDRE) as monitored by gel mobility shift analysis. Thus, we conclude that unmodified serine 51 and its surrounding basic residues are crucial not only for PKC-β substrate recognition but also for the optimal VDRE binding of native hVDR. In transactivation assays, S51G and S51D possessed only 35 and 10% of wild-type hVDR activity, respectively. Mutation of serine 51 to threonine (S51T) restored phosphorylation by PKC-β, *in vitro*, to about 40% of wild-type and transactivation to 45% of that of wild-type hVDR. Alteration of serine 51 to alanine, which is the residue in the corresponding position of the glucocorticoid, progesterone, mineralocorticoid, and androgen receptors, eliminated PKC-β phosphorylation but completely preserved the specific DNA binding activity and transactivation capacity of hVDR. Thus, phosphorylation of hVDR at serine 51 is not required for either VDRE binding or transactivation. Finally, incubation of *Escherichia coli*-expressed hVDR with PKC-β elicits marked phosphorylation of the receptor and significantly inhibits its ability to complex with the VDRE. We therefore speculate that posttranslational modification of hVDR at serine 51 may constitute a negative regulatory loop which could be operative when target cells are subject to PKC activation events.

The vitamin D receptor (VDR)[1] is classified as a member of the steroid/thyroid hormone receptor superfamily of proteins by virtue of amino acid homologies and similar biochemical functions among this family of nuclear regulatory macromolecules (Beato, 1989; Evans, 1988; Haussler *et al.*, 1988). As with other steroid/thyroid/retinoid receptors, the intranuclear VDR mediates the biological effects of the vitamin D hormonal ligand, 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$), on target cells. Hormone-occupied VDR regulates specific gene transcription by binding to a DNA enhancer sequence termed the vitamin D-responsive element (VDRE). Recently, several VDRE sequences have been identified in the mouse *Spp-1*/osteopontin gene (Noda *et al.*, 1990), the human osteocalcin gene (Ozono *et al.*, 1990), and the rat osteocalcin gene (DeMay *et al.*, 1990; Markose *et al.*, 1990; Terpening *et al.*, 1991). Guanine nucleotide contact sites for VDR within the VDRE sequence have been mapped and the requirement for both nuclear protein auxiliary factor(s) and 1,25-(OH)$_2$D$_3$ for the interaction of VDR with the VDRE has been characterized via gel retardation analyses (Ozono *et al.*, 1990; MacDonald *et al.*, 1991; Nakajima *et al.*, 1992). Therefore, like the thyroid hormone and retinoic acid receptors, gene control by VDR is apparently elicited by binding of a VDR: auxiliary factor heterodimer to direct repeats of the VDRE half-element which are separated by 3 base pairs in vitamin D-controlled genes (Haussler *et al.*, 1991; Sone *et al.*, 1991; Umesono *et al.*, 1991).

Most of the steroid/thyroid receptors are known to be phosphorylated (Ortí *et al.*, 1992), and specific phosphorylated residues have been identified in the mouse glucocorticoid receptor (Bodwell *et al.*, 1991), the chicken progesterone receptor (Denner *et al.*, 1990a, 1990b), and the chicken thyroid hormone receptor (Goldberg *et al.*, 1988; Glineur *et al.*, 1989). Although phosphorylation of proteins is widely regarded as one of the key biochemical means of regulating cellular processes, the functional role for phosphorylation of steroid/thy-

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[1] The abbreviations used are: VDR, vitamin D receptor; 1,25-(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; VDRE, vitamin D-responsive element; hVDR, human VDR; PKC-β, protein kinase C-β; DMEM, Dulbecco's modified Eagle's medium; hGH, human growth hormone; PBS, Dulbecco's phosphate-buffered saline; TRβ, thyroid hormone receptor-β; RRα, retinoic acid receptor-α; ER, estrogen receptor; GR, glucocorticoid receptor; PR, progesterone receptor, MR, mineralocorticoid receptor; AR, androgen receptor.

roid receptors is not clearly defined. Recent evidence indicates that phosphorylation of the chicken progesterone receptor may be centrally involved in the transcriptional activation function (Denner *et al.*, 1990b). It is therefore of considerable importance to characterize the phosphorylation of steroid receptors and to understand its role in hormone receptor action.

Previous studies showed that the phosphorylation of mouse and chicken VDR, *in vivo*, is a rapid event which is enhanced in the presence of 1,25-$(OH)_2D_3$ (Pike and Sleator, 1985; Haussler *et al.*, 1988; Brown and DeLuca, 1990). Serine is the predominant phosphorylated residue based upon phosphoamino acid analysis of mouse VDR (Haussler *et al.*, 1988) and of other steroid receptors (Smith *et al.*, 1989; Sheridan *et al.*, 1989). We have recently observed that the serine 51 residue of human VDR (hVDR), which is conserved in all known VDRs, is selectively phosphorylated by protein kinase C-β (PKC-β), *in vitro* and *in vivo* (Hsieh *et al.*, 1991). The family of PKC serine/threonine kinases has been well characterized, and its members are thought to play a crucial role in the signal transduction pathways elicited by a variety of growth factors, hormones, and neurotransmitters (Nishizuka, 1989), as well as in the modulation of transacting factors such as c-*jun* (Boyle *et al.*, 1991). Our earlier data revealed that the replacement of serine 51 in hVDR with glycine results in significant inhibition of transcriptional activation capacity. Thus, serine 51 warrants further study as a key residue in the phosphorylation and function of hVDR.

To further delineate the potential functional involvement of PKC phosphorylation in VDR action, we used oligonucleotide-directed mutagenesis to replace several amino acids at or around the PKC site. The effects of these mutations on protein expression, hormone binding, DNA binding, transactivation, and PKC-β phosphorylation are described in this paper. In addition, we evaluated the potential role of PKC phosphorylation of hVDR in terms of proportion of total hVDR phosphorylation, dependence on the presence of 1,25-$(OH)_2D_3$ hormone, and flanking sequence requirements for PKC-β recognition of serine 51. We observed that hVDR phosphorylation at serine 51 is a quantitatively significant modification in intact cells that apparently is unaffected by 1,25-$(OH)_2D_3$ hormone levels. The basic region, including serine 51, proved to be essential for specific binding of hVDR to DNA and possibly plays a minor role in nuclear localization. The introduction of a negative charge at residue 51 inhibits DNA binding, indicating that the function of PKC phosphorylation may be to down-regulate VDR action.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Alteration of specific residues in hVDR was carried out according to the method of Kunkel *et al.* (1987) using the MutaGene kit (Bio-Rad). Briefly, the hVDR cDNA was inserted into the *Eco*RI site of the phagemid vector pSG5 (Green *et al.*, 1988), which contains an M13 phage origin of replication. Single-stranded phagemid containing the hVDR cDNA was then produced and annealed to oligonucleotides complementary to the region of interest in the hVDR sequence. These oligonucleotides, usually 21–25 nucleotides in length, contained bases in the central portion of their sequence which mismatched with the natural hVDR sequences, creating altered amino acid codons. After annealing of these mutagenic oligonucleotides, the second strand of the pSG5hVDR phagemid was completed using T4 DNA polymerase in an *in vitro* synthesis. Double-stranded phagemid was then propagated in a bacterial host and isolated colonies were screened by DNA sequencing for the presence of pSG5hVDR phagemids containing the desired mutation.

Cell Culture and Transfections—COS-7, an SV40-transformed African Green monkey kidney cell line, was obtained from the American Type Culture Collection (Bethesda, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA). Cells were transfected using the calcium phosphate-DNA coprecipitation technique without glycerol shock (Kingston, 1990). Eighteen hours after transfection, cells were washed twice with DMEM and incubated in either the presence or absence of 10 nM 1,25-$(OH)_2D_3$ hormone for an additional 48 h prior to harvesting.

Transcription Assay—Transcriptional activity of hVDR mutants was measured in COS-7 cells cotransfected with the appropriate pSG5hVDR expression plasmid (7.5 μg) and a reporter plasmid (CT4)$_3$TKGH (5 μg) containing the vitamin D-responsive element (Terpening *et al.*, 1991). Cells were treated for 48 h following transfection with either 10 nM 1,25-$(OH)_2D_3$ or ethanol as a control. Medium was assayed for the expression of human growth hormone (hGH) by radioimmunoassay using a commercial kit (Nichols Institute Diagnostics, San Juan Capistrano, CA), and cells were harvested for immunoblot analysis.

Hormone Binding Assay—The abilities of wild-type and mutant hVDRs to bind 1,25-$(OH)_2D_3$ ligand were assessed by a filter binding assay as described by Jones *et al.* (1991), except that acetone treatment of filters was omitted.

Immunoblotting and Immunoprecipitation of Human VDR—Transfected COS-7 cells were lysed directly in 2% SDS, 5% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8, and 20% glycerol, and 40 μg of cellular protein was run on 5–15% gradient SDS-polyacrylamide gels. After electrophoretic fractionation, proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Transblot apparatus (Bio-Rad) in 25 mM Tris-HCl, pH 7.4, 192 mM glycine, 0.01% SDS, and 20% methanol. Immunodetection of bound hVDR proteins was then performed using the 9A7γ monoclonal anti-VDR antibody (Pike *et al.*, 1983). After the first antibody treatment, the Immobilon-P membrane was washed and treated at room temperature for 1 h with goat anti-rat IgG conjugated to biotin. After four 15-min washes, the blot was incubated with avidin-alkaline phosphatase for 1 h and then was washed four more times, followed by a fifth wash with biotin blot buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100). Finally, the blot was exposed to color reagent containing 50 μg/ml of 5-bromo-4-chloro-3-indolylphosphate and 100 μg/ml of 4-nitro blue tetrazolium chloride. The color reaction was stopped by washing with distilled water. The immunoblots shown in Figs. 4*B*, 5*C*, and 6*B* were generated using the above described method, whereas those in Figs. 1*B* and 7*B* were produced by the method described previously by Terpening *et al.* (1991) which uses ^{125}I-labeled protein A.

For immunoprecipitation, transfected COS-7 cells were lysed in immunoprecipitation lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.3 mM $ZnSO_4$, 0.3 M KCl, 0.5% Triton X-100) and incubated with monoclonal antibody 4A5γ linked to agarose beads as described elsewhere (Jones *et al.*, 1991).

Purification of PKC-β and Phosphorylation Reactions—Purification of PKC from mouse brain (Samuels and Shimizu, 1992) and *in vitro* PKC phosphorylation reactions of transfected COS-7 cell extracts were performed by standard procedures as described previously (Hsieh *et al.*, 1991). Recently, hVDR has been successfully overexpressed and purified from *Escherichia coli* cultures (Hsieh *et al.*, 1992). Crude and purified, *E. coli*-expressed hVDRs were evaluated as substrates for PKC-β by incubating them with 1 unit of the kinase for 30 min at room temperature in the presence of 30 μCi of [γ-^{32}P]ATP. To test the influence of PKC-β-catalyzed phosphorylation on the DNA binding activity of the receptor, purified *E. coli*-expressed hVDR (200 ng) was incubated with different amounts of PKC-β and 1 mM nonradioactive ATP in solution at room temperature for 50 min followed by either gel mobility shift assay or immunoblotting.

Gel Retardation Assay—A synthetic oligonucleotide, 5′-AGCTGC ACTGGGTGAATGAGGACATTACT-3′, containing the vitamin D-responsive element sequences of the rat osteocalcin gene (Terpening *et al.*, 1991; MacDonald *et al.*, 1991) was used as a probe in gel mobility shift assays. This double-stranded oligonucleotide, designated CT5, was labeled with [α-^{32}P]dCTP (3000 Ci/mmol, Du Pont-New England Nuclear) at 5′-overhanging ends with Klenow fragment. The hVDR utilized for these gel mobility shift assays was obtained from whole cell extracts of COS-7 cells transfected with wild-type or mutant pSG5hVDR phagemids. The cells were scraped, washed three times with phosphate-buffered saline (PBS: 136 mM NaCl, 26 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2), and resuspended in KETD-0.3 buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 300 mM KCl, 10% glycerol, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 15 μg/ml aprotinin, 1 mg/ml leupeptin, 1 μg/ml pepstatin A). After sonication, samples were centrifuged at 215,000 × *g* for 30 min at 2 °C. The supernatant was collected, divided into small aliquots, and stored at −70 °C. Rat liver nuclear extract, which alone does not form complexes with the VDRE (Nakajima *et*

al., 1992), containing 1 μg of protein, was preincubated with the hVDR-containing, whole cell extract (1 μg of total protein) in DNA binding buffer (10 mM Tris-HCl, pH 7.6, 100 mM KCl, 2 μg of bovine serum albumin, 1 μg of poly(dI·dC)) for 15 min at 22 °C and then incubated with 0.5 ng of ^{32}P-labeled probe for an additional 15 min. The reaction mixtures were loaded onto 4% nondenaturing polyacrylamide gels in 22.5 mM Tris borate, pH 7.2, 0.5 mM EDTA. Gels were run at 10 mA for 70 min, dried, and exposed for autoradiography.

Incubation of Intact Cells with Vitamin D Hormone and Cell Fractionation—At 48 h posttransfection, a total of 2×10^7 COS-7 cells were washed with DMEM and treated for 2 h at 37 °C with a final concentration of 10 nM 1,25-$(OH)_2D_3$. Cells then were harvested by scraping culture dishes, resuspending in ice-cold PBS, and centrifugation. The washed cell pellet was suspended in 1.5 ml of swelling buffer (0.1 M Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 5 mM dithiothreitol), incubated on ice for 10 min, and then disrupted by Dounce homogenization with a tight-fitting pestle. The homogenate was subjected to centrifugation at $1100 \times g$ for 15 min at 4 °C; the resultant supernatant is referred to as "cytosol extract." The crude nuclear pellet was suspended in 0.5 ml of KETD-0.3 buffer and extracted for 30 min. Soluble nuclear proteins, referred to as "nuclear extract," were obtained by centrifugation of the nuclear suspension at $27,000 \times g$ for 10 min. Cytosol and nuclear extract solutions were adjusted to equal protein concentrations and then analyzed for hVDR by immunoblotting (as described above) in order to estimate the subcellular partitioning of the transfected wild-type and mutant hVDRs. Although the absolute distribution of hVDR between cytosol and nucleus can only be obtained by methods such as protein immunofluorescence, this preliminary examination of hVDR-specific immunoreactivity provides some index of the nuclear retention of the receptor and its mutant forms.

RESULTS

Phosphorylation of hVDR on Ser^{51} in Intact Cells—As depicted in Fig. 1*A*, when an hVDR expression plasmid is transfected into COS-7 cells, the hVDR protein is significantly phosphorylated during a 4-h exposure of the cells to [^{32}P]orthophosphate. Mutation of serine 51 to glycine (S51G) abolishes approximately 60% of the phosphorylation of wild-type hVDR based upon quantitative densitometric scanning of the ^{32}P-VDR images (Fig. 1*A*, *lane 1 versus 3* and *lane 2 versus 4*), indicating that PKC-catalyzed phosphorylation of serine 51 constitutes a significant proportion of the phosphorylation of hVDR in intact cells. Fig. 1*B* depicts immunoblot analysis of wild-type and mutant receptor expression in this experiment; virtually equal expression occurs which argues that the differences in phosphorylation are not the result of variable expression or stability of the hVDR mutant. In addition, when cells expressing the wild-type and S51G hVDRs are exposed to 1,25-$(OH)_2D_3$, phosphorylation is only slightly stimulated in both cases. Based upon quantitative densitometric scanning of the ^{32}P-VDR image, an 11% increase in wild-type phosphorylation occurred in the presence of 1,25-$(OH)_2D_3$ when normalized to the level of hVDR expression (compare *lanes 1* and *2*); a 32% increase was seen in the case of the S51G mutant (compare *lanes 3* and *4*). The stimulation in the presence of sterol ligand is blunted in this particular experiment by the high level of hVDR, because in transfected cells with lower levels of hVDR expression, the stimulation of phosphorylation by 1,25-$(OH)_2D_3$ is as high as 2-fold (McDonnell *et al.*, 1989; Jones *et al.*, 1991; Jurutka *et al.*, 1993). Nevertheless, because alteration of the serine 51 PKC site does not diminish the slight increase in hVDR phosphorylation occurring in the presence of 1,25-$(OH)_2D_3$, this suggests that PKC-mediated phosphorylation of hVDR is independent of the presence of the 1,25-$(OH)_2D_3$ hormone.

Construction of Additional hVDR Mutants—To probe further the structure-function relationship of PKC phosphorylation of hVDR, a series of hVDR mutants in addition to S51G was generated using the pSG5hVDR plasmid (Hsieh *et al.*, 1991). These hVDRs, which were altered at or in the vicinity of serine 51, are illustrated in Fig. 2, and their identity was confirmed by DNA sequencing in all cases. To examine the influence of the flanking basic residues on PKC phosphorylation of hVDR at serine 51, one double mutant, R49WR50G, and one triple mutant, K53QR54GK55E, were generated. These two mutants convert the amino-terminal flank of serine 51 from Arg-Arg to Trp-Gly and the carboxyl terminal flank of serine 51 from Met-Lys-Arg-Lys to Met-Gln-Gly-Glu, respectively. To confirm the general importance of this basic domain between the zinc fingers of hVDR, an internally deleted mutant, ΔRRSMKRK, was constructed. Finally, to evaluate the possibility that an alteration in the first zinc

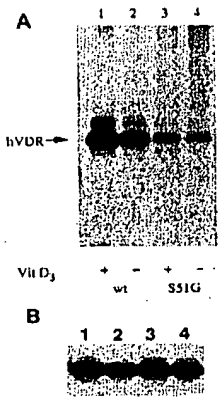

FIG. 1. **Phosphorylation of wild-type and mutant S51G hVDRs in intact cells.** *A*, COS-7 cells were transfected with either wild-type (*lanes 1* and *2*) or S51G mutant (*lanes 3* and *4*) hVDR cDNA, labeled for 4 h at 37 °C with [^{32}P]orthophosphate in the absence or presence of 10 nM 1,25-$(OH)_2D_3$, lysed, and subjected to immunoprecipitation. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. *B*, expression of wild-type and S51G hVDRs determined by immunoblotting of lysates from cells transfected as in *A*.

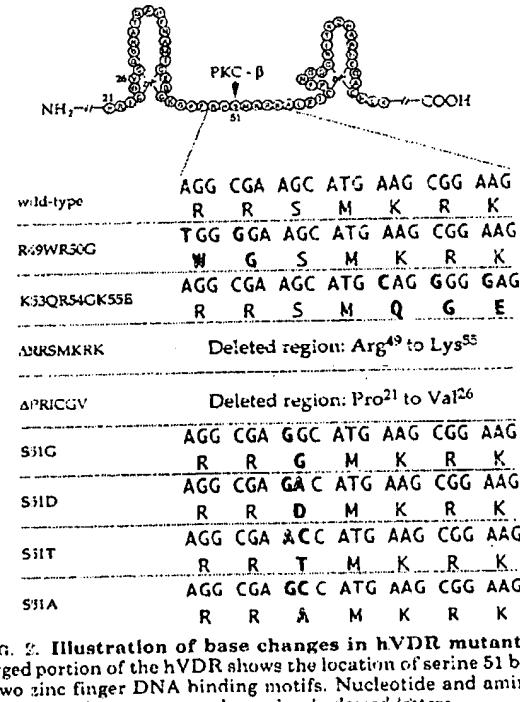

FIG. 2. **Illustration of base changes in hVDR mutants.** The enlarged portion of the hVDR shows the location of serine 51 between the two zinc finger DNA binding motifs. Nucleotide and amino acid changes in each mutant are shown in *shadowed letters*.

finger of the DNA binding region might affect PKC phosphorylation of hVDR, a deletion mutant, ΔPRICGV, in which 6 amino acid residues were deleted from the NH_2-terminal portion of the first finger (between Pro^{21} and Val^{26}), was generated. This PRICGV sequence displays high homology with a region in human and rat collagenase and stromelysin proteins (Vallee and Auld, 1990) and may be involved in the binding of a zinc atom.

In addition to the S51G point mutation, a constitutive negative charge was created by replacing serine 51 with aspartic acid (S51D). Threonine also was substituted for serine at position 51 to determine if this phosphorylatable amino acid could restore the wild-type activities of serine 51. Comparison of the sequences of the members of the nuclear receptor superfamily reveals that the thyroid hormone receptor (TR), retinoic acid receptor (RR), and the estrogen receptor (ER) possess a potentially phosphorylatable serine or threonine in the position analogous to hVDR serine 51, whereas in the glucocorticoid receptor (GR) subfamily this residue is an alanine. To gain insight into the structure-function relationships at the PKC site between these two subfamilies, and because alanine maintains α-helical structures well (Chakrabarty *et al.*, 1991; Serrano *et al.*, 1992), we constructed a serine 51 to alanine mutant (S51A).

PKC-β Catalyzed Phosphorylation of hVDR Mutants, in Vitro—Expressed hVDR mutants were immunoprecipitated and incubated with PKC-β in the presence of [γ-^{32}P]ATP, and the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography and quantitative densitometric scanning. Duplicate plates were lysed directly in buffer containing 2% SDS and 5 mM β-mercaptoethanol and subjected to Western blot analysis to monitor expression of each hVDR mutant (data not shown). As shown in Fig. 3, the 52-kDa polypeptides comprising wild-type (*lanes* 2 and 9) and S51T (*lane* 4) hVDRs were phosphorylated by PKC-β, although S51T hVDR retained only partial PKC-β phosphorylation (45% of wild-type hVDR when scanned densitometrically and normalized to expression). The S51G (*lane* 5) and S51D (*lane* 3) mutant receptors showed ≤5% of wild-type phosphorylation and the S51A mutant also lacked detectable PKC-mediated phosphorylation (data not shown). Mock transfection (*lane* 1) resulted in <2% of wild-type hVDR phosphorylation activity, demonstrating that the 52-kDa phosphopolypeptide resulting from the transient transfection of pSG5hVDR expression vectors in COS-7 cells represents an authentic hVDR species.

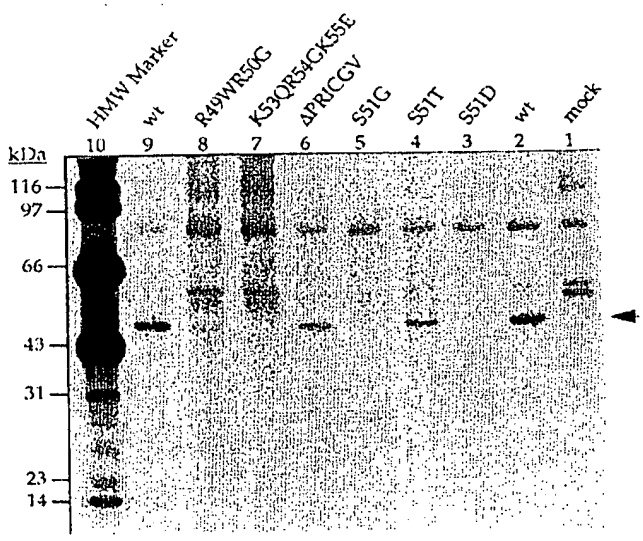

FIG. 3. **The role of position 51 and its flanking residues in the PKC-β-catalyzed phosphorylation of immunopurified hVDR.** COS-7 cells were transfected with hVDR constructs as indicated, or the pSG5 vector alone (*lane* 1), lysed, and immunoprecipitated. Antibody-bound hVDRs were then incubated with highly purified PKC-β. The apparent molecular mass of human VDR (52 kDa) is indicated by an *arrow*. When corrected for expression as determined by quantitative scanning of immunoblots, the relative phosphorylation intensities of mutant hVDRs compared with that of wild-type receptor (average of *lanes* 2 and 9) is: *lane* 1, 1.3%; *lane* 3, 2.6%; *lane* 4, 45.0%; *lane* 5, 5.0%; *lane* 6, 39.5%; *lane* 7, 2.5%; *lane* 8, 6.2%.

The ΔPRICGV mutation (*lane* 6) partially inhibited PKC catalyzed hVDR phosphorylation, down to 40% of wild-type after normalization to expression, suggesting that an intact conformation of the first zinc finger apparently is required for optimal substrate recognition of the nearby serine 51 substrate site by the PKC-β enzyme. In a previous report (Hsieh *et al.*, 1991), we showed that alteration of serines 7, 9, 119, or 125 did not influence the level of hVDR phosphorylation by PKC-β; these mutations provide a negative control to demonstrate that single alteration of selected amino acids in the NH_2-terminal domain does not compromise serine 51 phosphorylation via gross changes in secondary or tertiary structure. To examine the influence of residues in the immediate vicinity of serine 51 on PKC phosphorylation of hVDR, we tested mutants in which the flanking positively charged amino acids were modified to uncharged or negatively charged residues. Based upon densitometric scanning and normalization to expression, the K53QR54GK55E triple mutation (Fig. 3, *lane* 7) dramatically reduces PKC-β phosphorylation to <3% of wild-type, whereas the R49WR50G double mutant (*lane* 8) possesses only 6% of wild-type phosphorylation by PKC-β. These results confirm that positively charged residues on both the NH_2- and COOH-terminal sides of a serine contribute to the recognition motif for PKC (Kennelly and Krebs, 1991).

DNA Binding Activity of hVDR Mutants—Preliminary results (Hsieh *et al.*, 1991) on the binding of the S51G mutant hVDR to DNA via VDRE affinity chromatography proved to be variable and not reproducible. Therefore, to evaluate the DNA binding activity of wild-type and mutant hVDRs, a gel mobility shift assay in the presence of a mammalian cell nuclear extract was utilized to probe hVDR·VDRE complexes formed when whole cell extracts of transfected COS-7 cells were incubated with an authentic VDRE probe (CT5). After incubating ^{32}P-labeled CT5 oligonucleotides with whole cell extracts from wild-type hVDR-transfected cells and resolving the products on nondenaturing polyacrylamide gels, two major complexes were observed (Fig. 4, *lane* 10). These two complexes are not present in mock-transfected cells (*lane* 12), and their formation can be inhibited by the specific anti-VDR monoclonal antibody 9A7γ (*lane* 11). In addition, these complexes were competed by an excess of an unlabeled oligonucleotide CT5 (*lanes* 8 and 9). These data confirm that the two DNA-protein complexes represent specific high-affinity hVDR-VDRE interactions. Under the same conditions, S51T (*lane* 4) and S51A (*lane* 7) hVDRs exhibited wild-type DNA binding activity, whereas the S51G (*lane* 5) and S51D (*lane* 6) hVDRs did not show detectable binding to the VDRE. The basic region R_{49}RSMKRK$_{55}$ is strictly required for the DNA binding of hVDR, because neither the internally deleted mutant, ΔRRSMKRK (*lane* 3), nor the R49WR50G (*lane* 2) and K53QR54GK55E (*lane* 1) mutants, displayed any complex formation with the VDRE. These differences in DNA binding activity were not the result of variable expression of the mutant hVDRs, because Western blot analysis showed similar expression (Fig. 4*B*). From these observations, we conclude that: (i) the alteration of serine 51 to residues other than

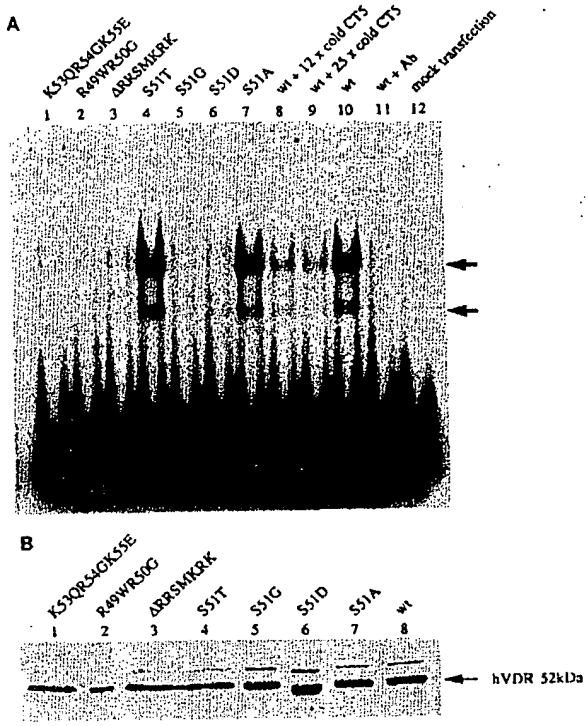

FIG. 4. **DNA binding analysis of wild-type and mutant hVDRs by gel mobility shift assays.** *A*, a ^{32}P-labeled CT5 oligonucleotide (0.5 ng) containing the VDRE from the rat osteocalcin gene was incubated with rat liver nuclear extract (containing 1 μg of protein) and the whole cell extract (containing 1 μg of protein) from COS-7 cells transfected with wild-type (*wt*) or mutant hVDRs as indicated. *Lane 12* illustrates the reaction with extract from mock transfected cells plus rat liver nuclear extract. *Lane 11* shows a preincubation of wild-type hVDR extract with a monoclonal anti-VDR antibody prior to incubation with the CT5 probe. *Lanes 8* and *9* show prior incubation of wild-type hVDR with 12- or 25-fold molar excess unlabeled CT5 for 15 min at room temperature. *Arrows* indicate the positions of hVDR·VDRE complexes. *B*, expression of hVDR determined by immunoblot analysis. Ten μg of protein equivalents of each cell extract was loaded onto a 10% SDS-polyacrylamide gel, followed by Western blot analysis as described under "Experimental Procedures." The *arrow* indicates the migration position of hVDR at 52 kDa.

threonine and alanine, which are the conserved amino acids at this position in the known steroid/retinoid/thyroid hormone receptors (see Fig. 8), abolishes the DNA binding activity of hVDR, (ii) the basic residues flanking serine 51 are absolutely required for hVDR DNA binding, and (iii) the presence of a constitutive negative charge in the position of serine 51 (S51D) precludes DNA binding and thus intimates that PKC-catalyzed phosphorylation of hVDR may negatively regulate its DNA binding activity.

PKC-β Phosphorylates E. coli-expressed hVDR and Inhibits its DNA Binding, in Vitro—Recently, we have successfully overexpressed hVDR in *E. coli* and purified the protein to near homogeneity (Hsieh *et al.*, 1992). First, we determined whether *E. coli*-expressed hVDR, like that expressed in COS-7 cells (see Fig. 3, *lanes* 2 and *9*), is an effective substrate for PKC-β phosphorylation. To examine PKC-β phosphorylation of *E. coli*-derived receptor, the expressed hVDR was incubated with PKC-β and [γ-^{32}P]ATP in solution, immunoprecipitated, and subjected to 10% SDS-polyacrylamide gel electrophoresis. The results shown in Fig. 5*A*, *lanes 1* and *3*, demonstrate that both crude and purified *E. coli*-expressed hVDRs are sub-

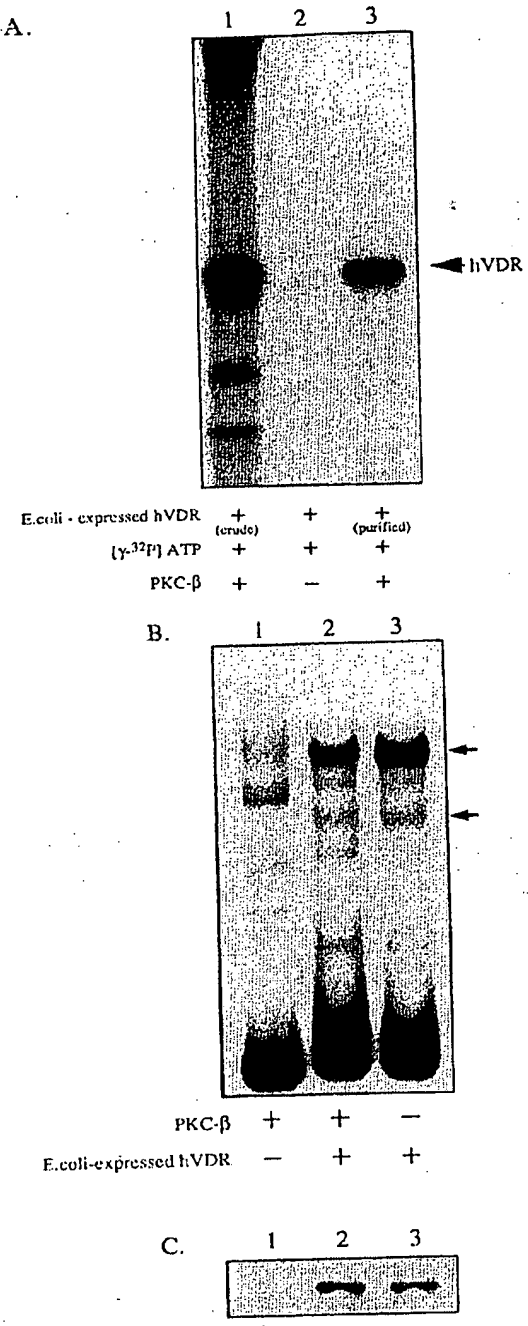

FIG. 5. **PKC-β phosphorylates E. coli-expressed hVDR and inhibits its DNA binding activity, *in vitro*.** *A*, phosphorylation of *E. coli*-expressed full-length hVDR by PKC-β, *in vitro*. Crude (*lane 1*) and purified (*lane 3*) *E. coli*-expressed hVDRs (2 μg) were incubated with PKC-β and [γ-^{32}P]ATP in solution at room temperature for 30 min, immunoprecipitated, and subjected to 10% SDS-polyacrylamide gel electrophoresis. *Lane 2* represents a purified hVDR incubation without PKC-β. *B*, PKC-β phosphorylation inhibits hVDR binding to the VDRE, *in vitro*. The purified hVDR (200 ng) was preincubated with 1 mM ATP in the presence or absence of PKC-β (5 units) at room temperature for 50 min and then evaluated by gel mobility shift assay in the presence of rat liver nuclear extract as described in Fig. 4*A* to determine the effect of PKC phosphorylation on DNA binding. *Arrows* indicate the positions of hVDR·VDRE complexes. *C*, quantitation of hVDR determined by immunoblotting in the presence (*lane 2*) or absence (*lane 3*) of PKC-β.

strates for PKC-β phosphorylation, *in vitro*. In the absence of PKC-β, there was no phosphorylated band apparent at the position of hVDR (Fig. 5*A*, *lane 2*). These results indicate *E. coli*-expressed hVDR retains the native conformation of the PKC-β recognition site and also that no endogenous PKC-β or other VDR kinase activity exists in *E. coli* preparations. To investigate next the possibility that introduction of a negative charge in the position of serine 51 may attenuate hVDR DNA binding, purified *E. coli*-expressed hVDR was preincubated at room temperature for 50 min in the presence or absence of PKC-β, and the ability of the receptor to associate with the VDRE was assessed by gel mobility shift assay. As shown in Fig. 5*B*, treatment with PKC-β appears to slightly attenuate specific DNA binding (compare *lanes 2* and *3*, Fig. 5*B*). Note that the occurrence of the shifted complex is dependent upon the inclusion of hVDR in the reaction (compare *lanes 1* and *3*, Fig. 5*B*). The observed DNA binding inhibition was not the result of differential hVDR degradation, as indicated by immunoblot analysis in this experiment (Fig. 5*C*). Because the reduction in hVDR·VDRE binding generated by incubation the receptor with PKC-β was modest with 5 units of kinase (Fig. 5*B*), we repeated this experiment with 0, 4, and 8 units to titrate the effect of the enzyme. Fig. 6 depicts the results of this experiment, and it is clear that there exists a concentration-dependent inhibitory effect of PKC-β treatment of hVDR on the ability of the receptor to retard the mobility of the labeled VDRE probe. Again, from the examination of immunoblots (Fig. 6*B*), this decrease in DNA binding activity was not the result of degradation of the hVDR protein. Finally, as illustrated in *lane 4* (Fig. 6*A*), the shifted complexes contain hVDR, because they are specifically decreased in the presence of anti-VDR monoclonal antibody 9A7γ, concomitant with the appearance of a supershifted band. These results are consistent with the observation that S51D hVDR exhibits a dramatic loss in DNA binding activity (Fig. 4) and suggest that one of the roles of PKC phosphorylation of hVDR is to attenuate interaction with the VDRE by phosphorylating serine 51.

Transactivation of a VDRE-Reporter Construct by hVDR Mutants--Transcriptional activation was tested in COS-7 cells cotransfected with mutant or wild-type pSG5hVDR expression vectors and a reporter plasmid containing four copies of the VDRE upstream of the herpes simplex virus thymidine kinase promoter directing the transcription of the hGH gene (Terpening *et al.*, 1991). After transfection into COS-7 cells, transactivation of the hGH gene was assayed by measuring the appearance of hGH in cell media. A comparison of transcriptional activity of the wild-type and mutant hVDRs is shown in Fig. 7*A*. The results illustrate that mutations S51T, S51G, and S51D elicited approximately 55, 65, and 90% inhibition of transcriptional activity, respectively. In

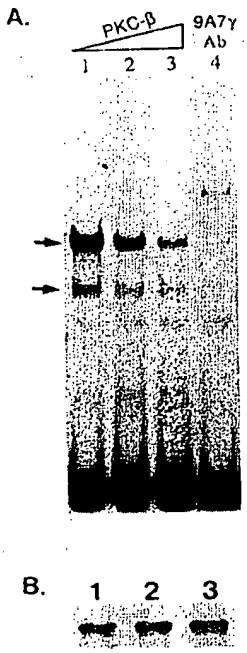

FIG. 6. **Effect of PKC-β phosphorylation on the binding of *E. coli*-expressed hVDR to the VDRE, *in vitro*.** *A*, purified *E. coli*-expressed hVDR (200 ng) was preincubated with 0 (*lane 1*), 4 (*lane 2*), or 8 (*lane 3*) units of PKC-β or hVDR-specific monoclonal antibody 9A7γ in the absence of PKC-β (*lane 4*) at room temperature for 50 min prior to a gel mobility shift assay. *Arrows* indicate the positions of hVDR·VDRE complexes. *B*, quantitation of hVDR by immunoblotting of the PKC-β-treated hVDR in *A*. *Lanes 1–3* represent the same order as in *A*.

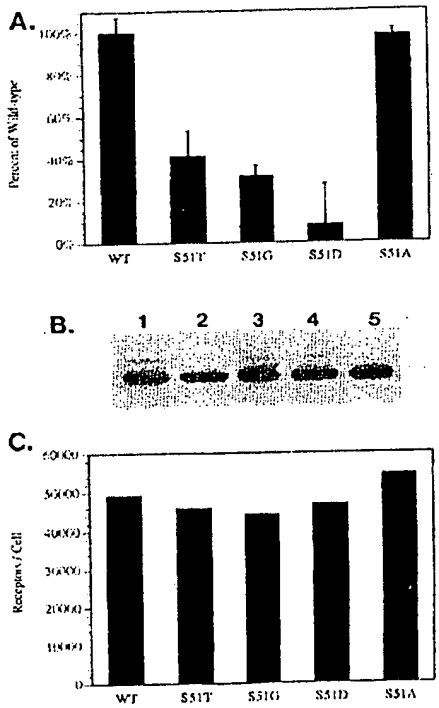

FIG. 7. **Functional assessment of hVDR mutants.** *A*, ability of transfected hVDRs to activate transcription of a vitamin D-responsive human growth hormone reporter gene construct. The indicated hVDR constructs (7.5 μg) were cotransfected into COS-7 cells along with the reporter plasmid (CT4)$_4$TKGH (5 μg) containing four copies of an intact vitamin D-responsive element inserted upstream of the thymidine kinase promoter. Assay of secreted hGH was performed as described under "Experimental Procedures." Results represent the means of at least three experiments (± S.E.) in which each assay was performed in duplicate. *B*, Western immunoblot analysis to assess expression of each hVDR protein. Whole cell extract containing 40 μg of protein was loaded onto each lane of the 10% SDS-polyacrylamide gel (*lane 1*, *WT*; *lane 2*, *S51T*; *lane 3*, *S51G*; *lane 4*, *S51D*; *lane 5*, *S51A*). Mock-transfected COS-7 cells possess undetectable hVDR levels by immunoblot analysis (data not shown). *C*, 1,25-(OH)$_2$[^{3}H]D$_3$ hormone binding activity measured by the filter-binding technique. The results are representative of at least three separate experiments. Untransfected COS-7 cells express the low level of approximately 1000 VDR molecules/cell when assayed by radioactive ligand binding (data not shown).

contrast, S51A retains wild-type hVDR transactivation capacity, and this result is consistent with its wild-type DNA binding activity. These findings suggest that secondary structure in the region of Ser^{51} may be more relevant to transactivation than phosphorylation of that residue, *per se*.

The retention of a small residual activity (~35%) by the S51G mutant in spite of nondetectable DNA binding (Fig. 4) may indicate a limitation in the conditions of the *in vitro* gel shift assay, such that results should be judged qualitatively as "all or none" rather than quantitatively. The transcriptional activation results are probably reflective of the S51G mutant's true character, as they were performed in intact cells, rather than in an *in vitro* setting.

Immunoblot analysis (Fig. 7*B*) confirmed that the observed transcriptional inhibition was not a consequence of a decrease in expression of any of the tested hVDR mutants. In addition, 1,25-$(OH)_2[^3H]D_3$ hormone binding values for wild-type and mutant receptors (Fig. 7*C*) indicate that pSG5hVDR-transfected COS-7 cells contain approximately 50,000 receptors/cell (200 pmol/mg of protein) in all cases. Equivalent hormone binding is not unexpected, since the mutations were introduced in the NH_2-terminal region and not in the COOH-terminal hormone binding domain of hVDR. Finally, to verify that the impaired transactivation potency of several of the mutant hVDRs was not the result of defective nuclear localization, nuclear and cytoplasmic fractions from cells transfected with mutant or wild-type hVDRs were examined separately for the presence of expressed hVDR by immunoblotting. The results revealed that all hVDRs evaluated were present in significant concentrations in both the nuclear and cytosolic fractions (data not shown). However, wild-type, S51T, and S51G hVDRs partitioned with a slightly higher specific activity in the nuclear fraction, whereas the S51D mutant hVDR, as well as mutants with flanking basic residues replaced on the either side of serine 51 (R49WR50G and K53QR54GK55E), existed in slightly higher specific activities in the cytosolic fraction (data not shown). Therefore, insertion of a constitutive negative charge at position 51, or eliminating either of the flanking positive amino acid clusters, apparently blunted the nuclear transfer of hVDR to a small degree. This suggests that the basic region between amino acids 49 and 55 is required for optimal nuclear retention of hVDR, but is neither the only nor the primary nuclear localization signal. Therefore, from all of the results above we conclude that the reduced transcriptional activities of S51T, S51G, and S51D are not the result of (i) variable protein expression/stability, (ii) different 1,25-$(OH)_2D_3$ binding activities, or (iii) significantly different partitioning to the nucleus.

Summary of Mutant hVDR Biochemical Properties—Table I presents a summary of the molecular characteristics of various mutant hVDRs altered in or around the serine 51 residue; subcellular partitioning is not included, because it was not determined quantitatively. Most of these mutations between the DNA binding fingers of hVDR compromise both the VDRE association and the transactivation capability of the receptor. The exception is S51A, which introduces an alanine residue characteristic of the glucocorticoid receptor and its closely related receptors. Many mutants lose DNA binding capacity in parallel with a reduction in transactivation, intimating that an attenuation of VDRE association accounts, in part, for the diminished transcriptional activity of these mutant hVDRs. PKC phosphorylation of residue 51 of hVDR does not correlate well with any of the tested parameters. It is markedly dependent upon basic residues surrounding serine 51, but apparently is not absolutely required for 1,25-$(OH)_2D_3$ hormone binding, VDRE binding, or transactivation. The fact that substitution of alanine at position 51 mimics wild-type hVDR activity argues that the secondary structure of this basic region of the receptor is more significant to function than is its phosphorylation by PKC. Therefore, the basic region between the two zinc fingers of hVDR may optimize nuclear localization and certainly participates in DNA binding, with the phosphorylation of serine 51 acting as a reversible negative signal to block the DNA binding ability of hVDR.

DISCUSSION

The present data extend our previous observation that transfected hVDR is a substrate for PKC, both *in vitro* and *in vivo*, and that the unique site of phosphorylation by this kinase is serine 51 (Hsieh *et al.*, 1991). In addition to the novel observation that *E. coli*-expressed hVDR is phosphorylated by PKC-β (Fig. 5*A*), we have also shown in a separate publication (Haussler *et al.*, 1991) that purified, baculovirus-expressed hVDR is phosphorylated efficiently by PKC-β, *in vitro*. Thus, there seems little doubt that hVDR is a substrate for PKC and that this reaction occurs in intact COS-7 cells (Fig. 1*A*). Although we are unaware of data on the relative expression of PKC in COS-7 cells, PKC-catalyzed phosphorylation of hVDR accounts for up to 60% of the basal phosphorylation of hVDR in these cells during a 4-h incubation, and this posttranslational modification apparently is not stimulated by exposure of the cells to the 1,25-$(OH)_2D_3$ hormonal ligand (Fig. 1*A*). However, we have previously demonstrated enhanced hVDR phosphorylation at serine 51 when CV-1 cells are treated with a phorbol ester activator of PKC (Hsieh *et al.*, 1991). Also consistent with PKC-mediated modification of hVDR is the finding that alteration of basic residues on either flank of serine 51 attenuates hVDR phosphorylation (Fig. 3), presumably by disrupting the recognition motif for the PKC enzyme, the optimal configuration of which consists of two separate positively charged regions that straddle the phosphorylated serine or threonine (see Kennelly and Krebs, 1991 and references therein).

The major focus of the current study was to further evaluate PKC phosphorylation of hVDR in terms of its biochemical

TABLE I

Summary of the phenotypes of wild-type and mutant hVDRs

Mutant	1,25-$(OH)_2D_3$ binding	Transactivation	DNA/VDRE binding[a]	PKC phosphorylation
Wild-type	++++[b]	++++	++++	++++
S51G	++++	++	−	−
R49WR50G	++++	+	−	+
K53QR54GK55E	++++	+	−	−
S51D	++++	+	−	−
S51T	++++	++	++++	++
S51A	++++	++++	++++	−

[a] Measured under the conditions of a gel mobility shift assay.
[b] Relative activity compared with wild-type is as follows: ++++, 75–100%; +++, 50–75%; ++, 25–50%; +, 5–25%; −, 0–5%.

function. Inactivation of the PKC phosphorylation site in hVDR by site-specific mutagenesis caused a spectrum of reductions in transcriptional activity (Fig. 7*A*; Table I), although no correlation between phosphorylatability and transcriptional activity was found. In fact, attempting to mimic the negative charge of phosphoserine at residue 51 by inserting an aspartic acid failed either to generate a constitutively active hVDR or to regenerate the transcriptional activity of wild-type hVDR; instead this substitution rendered the receptor a poor binder of DNA (Fig. 4*A*) and a very weak transactivator (Fig. 7*A*). Such a result is not without precedent, since both the CREB transacting protein (Gonzalez and Montminy, 1989) and the human androgen receptor (Kemppainen *et al.*, 1991) are known to be phosphorylated at specific residues, and yet their transacting capacities are not retained by the insertion of constitutively negatively charged amino acids. In the case of CREB, mutation of the serine 133 protein kinase A site to aspartic acid did not create a constitutively active transcription factor (Gonzalez and Montminy, 1989). Similarly, when serine 735 in the human androgen receptor, which is phosphorylated by an unknown kinase, is replaced with either glutamic or aspartic acid, both high affinity androgen binding and transactivation are lost (Kemppainen *et al.*, 1991). Therefore, the consequences of phosphorylation of transcription factors such as hVDR may be more complicated than the mere presence of a negative charge. Reversibility via phosphorylation/dephosphorylation could be essential to a cyclical mechanism of action. Alternatively, phosphorylation may mediate specific protein-protein interactions not simulated by the introduction of a negative charge. Phosphorylation could also influence the conformation of regions of the target protein either adjacent in the primary sequence, as is the case in CREB (Yamamoto *et al.*, 1990), or nearby in the tertiary structure. Finally, phosphorylation could represent a negative regulatory loop, as it does in the case of c-*myb* (Lüscher *et al.*, 1990) and of myogenin (Li *et al.*, 1992).

The relevance of serine 51 in hVDR can also be inferred from a comparison of the residues in this position in members of both subfamilies within the steroid/thyroid/retinoid hormone receptor superfamily (Beato, 1989). A phosphorylatable serine or threonine residue exists in the appropriate position in every member of the hVDR subfamily described to date, represented in Fig. 8 by the human receptors for vitamin D, retinoic acid, thyroid hormone, and estrogen. Indeed, a comparison of 20 members of the superfamily (Koelle *et al.*, 1991 and references therein), including several receptors whose ligand is unknown, indicated that 16 receptors contained a serine or threonine residue at the corresponding position, with the only exceptions being those four receptors in the GR subfamily (Koelle *et al.*, 1991 and Fig. 8, *bottom*), which have an alanine at this position. The widespread conservation of the phosphorylatable serine/threonine and of the nonphosphorylatable alanine argues that these residues perform some crucial function in their respective subfamilies, and our results (Fig. 4) have shown that all three of these conserved residues confer specific high affinity DNA binding activity upon hVDR.

hVDR Subfamily

hVDR	C	E	G	C	K	G	F	F	R	R	S	M	K	R	K	A	L	F	T	C	P
hRRα	C	E	G	C	K	G	F	F	R	R	S	I	Q	K	N	M	V	Y	T	C	H
hTRβ	C	E	G	C	K	G	F	F	R	R	T	I	Q	K	N	L	H	P	S	Y	S
hER	C	E	G	C	K	A	F	F	K	R	S	I	Q	G	H	N	D	Y	M	C	P

hGR Subfamily

hGR	C	G	S	C	K	V	F	F	K	R	A	V	E	G	Q	H	N	Y	L	C	A
hPR	C	G	S	C	K	V	F	F	K	R	A	M	E	G	Q	H	N	Y	L	C	A
hMR	C	G	S	C	K	V	F	F	K	R	A	V	E	G	Q	H	N	Y	L	C	A
hAR	C	G	S	C	K	V	F	F	K	R	A	A	E	G	K	Q	K	Y	L	C	A

FIG. 8. **Comparison of the regions surrounding the site of PKC phosphorylation in hVDR with the analogous sequences in other members of the steroid receptor superfamily.** The sequence from Cys[41] to residue 61 in hVDR is shown, and *boxed* residues indicate the position corresponding to serine 51 in hVDR. Sequences illustrated are from *top*: hVDR (Baker *et al.*, 1988); hRRα (Giguere *et al.*, 1987); hTRβ (Weinberger *et al.*, 1986); hER (Green *et al.*, 1986); hGR (Hollenberg *et al.*, 1985); hPR (Misrahi *et al.*, 1987); hMR (Arriza *et al.*, 1987); hAR (Lubahn *et al.*, 1988). The sequences depicted represent the COOH-terminal region of the first zinc finger.

Indeed, when we introduced an alanine, rather than a glycine, at position 51 of hVDR, corresponding to the residue present in the GR subfamily, the result was a receptor with essentially wild-type capacity for transactivation and DNA binding. Thus, alanine appears to preserve transcriptional activation and DNA binding by the hVDR, whereas glycine cannot, suggesting that some factor other than availability for phosphorylation may be important at this position. When we determined the predicted secondary structure of this region using Chou-Fasman computer routines (Devereux *et al.*, 1984), it was found that the residues in this region possess the potential to form an α-helix (data not shown). Structural determinations by NMR and x-ray crystallography for the GR (Härd *et al.*, 1990; Luisi *et al.*, 1991) and ER (Schwabe *et al.*, 1990) have demonstrated the existence of an α-helical structure extending from the 2 cysteines at the *left* of Fig. 8 through the position equivalent to residue 51 in the hVDR. In addition, it has recently been shown that alanine has a much greater propensity for maintaining an α-helix than does glycine (Chakrabartty *et al.*, 1991; Serrano *et al.*, 1992). Moreover, threonine is less able than serine to maintain this structure, possibly explaining why the S51T mutant does not fully restore transactivation capacity in intact cells. Taken together, these observations lead to the conclusion that an α-helix in this region is important for the transcriptional activation function of receptors in the superfamily and that insertion of a residue such as glycine which could disrupt this structure is detrimental. The effects of serine 51 phosphorylation on the α-helix are unknown and may warrant further investigation.

One preliminary finding in our experiments is that the region $RRS_{51}MKRK$ seems to contribute to nuclear localization of hVDR (data not shown). However, our data reflect overexpressed receptors, and it is likely that the high levels of hVDR required to carry out subsequent biochemical experiments such as phosphorylation reactions and gel mobility shift analysis cause saturation of nuclear sites with excess receptor partitioning to the cytosol. Therefore, more definitive studies employing immunocytochemical technologies with normal endogenous levels of hVDR and its mutant forms must be completed in order to draw firm conclusions. Nevertheless, this bipartite cluster of basic residues is most evident in hVDR as compared with other steroid receptors (Fig. 8). It resembles other nuclear translocation signals, although it does not fit well with one interpretation of such sequences which invokes a spacer of 10 amino acids between the basic regions (Dingwall and Laskey, 1991). However, an intriguing fact is that phosphorylatable serines or threonines very often exist within or adjacent to such basic nuclear localization sequences, tempting us to speculate that the rate of nuclear import of hVDR, like other nuclear proteins, is regulated by phosphorylation (see Dingwall and Laskey, 1991 and references therein). One could theorize that such a control by PKC would limit transactivation by 1,25-$(OH)_2D_3$ during certain periods of cell growth and differentiation.

Because of the location of serine 51 between the two zinc fingers and in the midst of a bipartite basic cluster, it is also likely that this region of hVDR plays a role in DNA binding. In fact, a natural mutation of arginine 50 to glutamine has

been reported in a patient with hereditary resistance to 1,25-$(OH)_2D_3$.[2] In this case the mutant receptor does not bind to DNA with high affinity. The R49WR50G mutant generated in the present work is analogous to that natural mutant in that it does not bind well to DNA (Fig. 4*A*; Table I). Introduction of a negatively charged aspartic acid at position 51 also renders hVDR a poor binder of the VDRE (Fig. 4*A*), which may be the result of charge repulsion by the phosphate backbone of DNA. Most importantly, we show here in direct experiments that reaction of *E. coli*-expressed hVDR with PKC-β, *in vitro*, dramatically diminishes the ability of the receptor to bind to the VDRE (Figs. 5 and 6). Precedents for inhibition of DNA binding by phosphorylation exist in the cases of c-*myb* (Lüscher *et al.*, 1990) and *max* (Berberich and Cole, 1992). Recently, myogenin, a helix-loop-helix protein that activates muscle cell transcription by binding to a conserved DNA sequence in many muscle-specific genes, has been found to be phosphorylated by PKC in its DNA binding domain that results in the repression of the myogenic program through loss of DNA binding activity (Li *et al.*, 1992). Although requiring further proof, we can extend this line of reasoning to speculate that the actual purpose of serine 51 phosphorylation of hVDR may be to effect a negative regulation of DNA binding, *in vivo*, and perhaps also to blunt nuclear uptake. This would provide a powerful "cross-talk" in which activators of PKC could silence hVDR and possibly other members of the hVDR subfamily in their ability to regulate specific gene transcription.

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[2] Sone, T., Scott, R., Kerner, S., Takeda, E., and Pike, J. (1993) *Eighth Workshop on Vitamin D, July 5–10, 1991, Paris, France*, Abstract. Arginine 50 is referred to in this abstract as arginine 47, a discrepancy based on the presence of two candidate initial methionines for hVDR located three positions apart (see Baker *et al.*, 1988).

Phorbol Ester Treatment Increases the Exocytic Rate of the Transferrin Receptor Recycling Pathway Independent of Serine-24 Phosphorylation

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Abstract. In Chinese hamster ovary (CHO) fibroblast cells the protein kinase C activating phorbol ester, phorbol myristate acetate (PMA), stimulates an increase in cell surface transferrin receptor (TR) expression by increasing the exocytic rate of the recycling pathway. The human TR expressed in CHO cells is similarly affected by PMA treatment. A mutant human TR in which the major protein kinase C phosphorylation site, serine 24, has been replaced with the non-phosphorylatable amino acid glycine has been constructed to investigate the role of receptor phosphorylation in the PMA induced up-regulation. The Gly-24-substituted receptor binds, internalizes, and recycles Tf. Furthermore, the altered receptor mediates cellular Fe accumulation from diferric-Tf, thereby fulfilling the receptor's major biological role. The Gly-24 TR behaves identically to the wild-type TR when cells are treated with PMA. Therefore, Ser-24 phosphorylation is not required for the PMA-induced redistribution of the human TR expressed in CHO cells. The increased TR expression on the cell surface after PMA treatment results from an increase in the rate of exocytosis of the recycling receptors. No change in the endocytic rate or the size of the recycling receptor pool was observed. These results indicate that the PMA effect on the TR surface expression may result from a more general perturbation of membrane trafficking rather than a specific modulation of the TR.

IRON uptake in mammalian cells is mediated by transferrin receptor (TR)[1] endocytosis. Diferric transferrin (Tf) binds to the TR and is internalized by receptor-mediated endocytosis. Tf releases iron upon encountering an acidic intracellular vesicle pool. The apoTf-TR complex is recycled back to the cell surface to mediate further rounds of endocytosis (for review see Hanover and Dickson, 1985, and Huebers and Finch, 1987). The features of the TR responsible for internalization and recycling have not been identified.

The steady-state TR distribution between intracellular pools and the cell surface is altered by treatment with polypeptide hormones and chemical mitogens (Buys et al., 1984; Davis and Czech, 1986; Davis et al., 1986*a*; Fallon and Schwartz, 1986; Klausner et al., 1984; May et al., 1984; May et al., 1985; Tanner and Lienhard, 1987; Wiley and Kaplan, 1984). The effect is cell line and treatment dependent. For example, in the K562 human erythroleukemia cell line phorbol 12-myristate 13-acetate (PMA) causes a rapid decrease in cell surface TR expression (Klausner et al., 1984), whereas in mouse tumor macrophage-like cells, J774, PMA causes an increase in cell surface TR expression (Buys et al., 1984). Since PMA treatment also induces a reversible hyperphosphorylation of the TR, it has been proposed that TR phosphorylation is involved in regulating the TR distribution (Klausner et al., 1984; May et al., 1984). The cytoplasmic Ser-24 residue of the human TR has been identified as the site of PMA-activated protein kinase C phosphorylation (Davis et al., 1986*b*). By in vitro site-directed mutagenesis of the cDNA clone of the human TR the role of TR phosphorylation in regulation of TR distribution can be investigated.

Two groups have recently shown that Ser-24 phosphorylation is not required for endocytosis or recycling of the TR (Rothenberger et al., 1987, Zerial et al., 1987). In both studies the TR was transfected into cell lines expressing endogenous receptor. To follow the behavior of the transfected receptor, in the background of endogenous receptor, it was necessary to either express high levels of the transfected TR (approximately 10-fold over endogenous levels) or to use specific anti-human-TR antibodies as a marker for the TR. In neither case was it possible to study the TR behavior using Tf as a marker in clonal cell lines expressing a typical number of TR.

To circumvent the difficulties presented by the endogenous TR background we have developed a heterologous system in which to study the behavior of in vitro mutated human TR (McGraw et al., 1987). We have isolated TR-variant Chinese hamster ovary (CHO) cells (termed TRVb cells) that, due to a defect in the endogenous TR, do not bind detectable amounts of Tf. We have used these cell lines as recipients of a cDNA clone of the human TR and have shown that the hu-

1. *Abbreviations used in this paper*: PMA, 12-phorbol 13 myristate acetate; Tf, transferrin; TR, transferrin receptor.

man TR expressed in TRVb cells behaves similarly to the hamster TR in wild-type CHO cells. The advantage of using this system for structure/function studies of the TR is that the behavior of the receptor can be characterized without interference from endogenous TR activity. This allows the use of Tf as a marker for the receptor in cell lines expressing a typical number of transfected TR. Furthermore, the biological function of the transfected receptors (that is, uptake of Fe via Tf) can be directly assessed without interference from endogenous receptor.

In this paper we report the characterization of the behavior of the human TR in which Ser-24 has been replaced with the nonphosphorylatable amino acid, Gly. The mutant receptor binds Tf, internalizes, and recycles similarly to the wild-type human receptor expressed in TRVb cells. We also show that PMA induces a rapid increase in cell surface expression of the hamster TR in CHO cells, as well as the human TR expressed in TRVb cells, by increasing the exocytic rate of the TR recycling pathway. Phosphorylation of Ser-24 is not required for the PMA effect since the Gly-24 substituted receptor is similarly affected by PMA treatment.

Materials and Methods

Cells and Cell Culture

WTB cells were used as the wild-type CHO cells (Thompson and Baker, 1973). The isolation and characterization of the TR-variant CHO cell line, TRVb, and the TRVb cell line transfected with the human TR, TRVb-1, have been described previously (McGraw et al., 1987). Cells were maintained in Ham's nutrient F12 medium (Gibco, Grand Island, NY) supplemented with 5% FCS at 37°C in a humidified atmosphere of 5% CO_2 in air. Transfectant cell lines were carried in medium containing 200 μg/ml G-418 (Gibco). Single cell colony lines were isolated by two rounds of cloning ring colony purification.

Ligands

Human Tf (Sigma Chemical Co., St. Louis, MO) was further purified by Sephacryl S-300 gel filtration. Differic Tf, fluorescein Tf, and [^{125}I]Tf were prepared as previously described (Yamashiro et al., 1984). $^{59}FeCl_3$ was purchased from New England Nuclear (Cambridge, MA). Tf$^{59}Fe_2$ was prepared by the nitrilotriacetic acid method (Klausner et al., 1984). Fluorescein-Tf uptake and immunofluorescence were performed as described previously (McGraw, et al., 1987). The human specific anti-TR monoclonal antibody B3/25 was purchased from Mannheim Biochemicals, (Indianapolis, IN).

Assays for PMA Effect on TR Expression

Approximately 1 × 10^5 cells were plated per 35-mm well of 6 well plates 2 d before use. For surface-binding studies the cell monolayers were washed three times with med 1 (150 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose), and incubated in med 1 for 30 min at 37°C in air. The cells were treated with 100 nM PMA (Sigma Chemical Co.) for specified periods of time. The cells were then washed twice with ice cold med 1, incubated with 2 μg/ml [^{125}I]Tf, 2 mg/ml ovalbumin at 4°C for 2 h. The cells were washed five times at 4°C with med 1, solubilized with 0.1% Triton X-100 in 0.1 N NaOH, and counted in a gamma counter. Nonspecific binding was determined by incubation in the presence of a 500-fold excess of unlabeled Tf. Nonspecific binding was generally less than 5% of total binding. Each experiment consisted of three experimental and three competition wells.

The Tf washout assay was performed by washing cell monolayers twice with med 1 and incubating with 2 μg/ml [^{125}I]Tf, 2 mg/ml ovalbumin for 60 min at 37°C. The cells were rapidly washed three times with med 1 containing 50 μM desferroxamine (Ciba-Geigy Corp., Greensboro, NC) and further incubated in med 1 containing 50 μM desferroxamine with or without 100 nM PMA. At the specified times the medium was aspirated and the cells were incubated on ice with 280 mM sucrose, 50 mM MES (pH 5.0) for 10 min. This acid wash insures that all surface bound [^{125}I]Tf is removed. The cells were then washed with ice cold med 1 and cell-associated radioactivity determined after solubilization of the monolayers. Desferroxamine was included in the incubations to ensure that apo-Tf released from the cell was not re-iron loaded.

Construction of Mutant TR

The ~4.9 kb Bam HI insert fragment of the cDNA clone of the human TR pCDTR1 (McClelland et al., 1984) was transferred to the Bam HI site of a pUC8 plasmid derivative in which the Hind III site had been eliminated. In this construct, pTM1008, the insert is oriented with the 5' end of the message adjacent to the Eco RI site of pUC8. pTM1008 contains two Hind III sites, one at 9II bp of the coding region of the insert and the other at ~3.8 kb of the non-coding region of the cDNA clone (McClelland et al., 1984). pTM1008 was partially digested with Hind III and the linearized fragment (~7.7 kb) was gel purified, the Hind III ends were filled-in by treatment with Klenow, blunt-end ligated and transformed into *Escherichia coli* strain JM109. Single colonies were purified and the DNA analysed by restriction digestion. A clone was selected in which the Hind III site in the non-coding region had been inactivated. This construct, pTM1010, retained the Hind III site in the coding region. The ~1 kb Eco RI (site in pUC8 sequences)-Hind III fragment of pTM1010 was transferred to Eco RI-Hind III digested M13mp18. This fragment contains the coding sequences for the NH_2 terminal 273 amino acids of the TR, including the 61 amino-acid NH_2 terminal cytoplasmic tail, the putative membrane-spanning region and 183 amino acids of the extracellular portion of the receptor. A synthetic oligonucleotide, 3'-GGGCCAAGCCGGACCG-5', in which the codon for Ser-24 has been replaced with the codon for Gly was used as primer for in vitro site-directed mutagenesis after the procedures of Zoller and Smith (1982) with the modifications of Kunkel (1985). The nucleotide change was confirmed by Sanger sequencing across the mutated site. The mutated Eco RI-Hind III fragment was isolated from the replicative form of M13 and was reinserted into pTM1010 replacing the wild-type Eco RI-Hind III fragment. Transfection of TRVb cells was performed as previously described (McGraw, et al., 1987). For transfection the Bam HI cDNA insert was mixed with Bam HI vector fragment of pCDTR1 and uncut pSV3-Neo. The cDNA plasmid, pCDTR1, only gives transfectants when the insert is released by Bam HI digestion (McGraw et al., 1987). We found that inclusion of the Bam HI vector fragment of pCDTR1 increases the TR positive transfection frequency. In this transfection scheme the promoter used for TR expression is unknown.

Results

PMA Effects an Increase in Cell Surface TR Expression in CHO Cells

Treatment of CHO cells with PMA causes a rapid increase in cell surface TR expression, as measured by an increase in [^{125}I]Tf binding at 4°C (Fig. 1). After a 5-min incubation with 100 nM PMA, [^{125}I]Tf surface binding increases by 30–50%. The effect is complete within 5 min, as longer incubations with PMA have no further effect on [^{125}I]Tf surface binding. Since CHO cells respond to PMA treatment by modulating the cell surface expression of the TR, these cells can be used as a recipient cell line for studying the role of phosphorylation in regulating cell surface TR expression.

We have isolated TR-variants of CHO cells (TRVb) which do not express detectable TR (McGraw et al., 1987). We have transfected a cDNA clone of the human TR into TRVb cells (the transfected cell line termed TRVb-1) and have shown that the transfected human TR behaves similarly to the hamster TR (McGraw et al., 1987). The cell surface expression of the human TR of TRVb-1 cells is rapidly increased after PMA treatment, in a fashion similar to the up-regulation of the hamster TR in CHO cells (Fig. 2 *a*). This PMA effect is dose-dependent, with a half-maximal dose of ~20 nM (Fig. 2 *b*). The PMA effect appears to be mediated by protein kinase C activation since treatment of TRVb-1 cells with 100

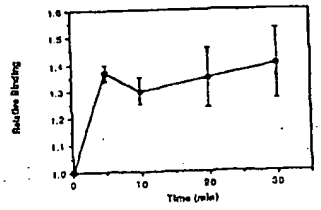

Figure 1. Time course of the PMA effect on cell surface TR expression in CHO cells. CHO cells were treated with 100 nM PMA in med 1 at 37°C for 5–30 min. The cells were cooled to 4°C, and [^{125}I]Tf-binding was measured. The results shown are the mean ± SEM of [^{125}I]Tf-binding relative to untreated cells in four separate experiments.

nM 4-alpha-phorbol, a phorbol ester which does not activate protein kinase C, did not alter the cell surface TR expression (not shown).

PMA treatment of TRVb-1 cells does not alter the affinity of the human TR for Tf. Scatchard analysis of [^{125}I]Tf 4°C binding to TRVb-1 cells treated for 10 min with 100 nM PMA revealed a 35% increase in surface binding with no significant change in the dissociation constant. In control cells the K_d was measured as 1.50 ± 0.1 nM; and after pretreatment with 100 nM PMA for 10 min the K_d was measured as 1.48 ± 0.02 nM K_d (mean ±SD of three experiments).

PMA Stimulates an Increase in the Exocytic Rate of the TR Recycling Pathway

Since the PMA effect on surface expression of TR is rapid it was unlikely that the effect was a result of the synthesis of new receptors. Rather, it was more likely that PMA was inducing a redistribution of existing TR. Surface expression could be increased by recruitment to the cell surface of intracellular TR either from the recycling pool of receptors (that is, altering the kinetics of recycling) or from a distinct pool of noncycling receptors (that is, increasing the size of the recycling pool). To distinguish between these possibilities the size of the recycling pool of TR was measured. TRVb-1 cells were incubated for 60 min at 37°C with 2 μg/ml [^{125}I]Tf to equilibrate the recycling pools of TR with [^{125}I]Tf. The cells were then incubated for an additional 15 min in 2 μg/ml [^{125}I]Tf with or without 100 nM PMA. The cells were washed and total cell-associated [^{125}I]Tf was determined. There was no difference in total cell-associated [^{125}I]Tf between cells treated with PMA and control cells. This result suggests that PMA is inducing a redistribution of TR in the recycling pool, since the total number of recycling TR was not increased by PMA treatment.

Increased cell surface TR expression could result from an increase in the exocytic rate (movement of intracellular receptors back to the cell surface) or a decrease in the rate of internalization. Since apo-Tf returns to the cell surface with the TR it is possible to characterize the rate of return of the TR to the cell surface by following the release of apo-Tf from the cell. To examine the effect of PMA treatment on the exocytic rate, cells were preincubated with [^{125}I]Tf to equilibrate the intracellular recycling pool of receptors with Tf. The cells were then washed free of unbound Tf and further incubated with or without 100 nM PMA. At various times the cells were washed to remove any released apo-Tf, and cell-associated radioactivity was determined. PMA treatment results in an increase in the rate of externalization of apo-Tf (Fig. 3). The calculated rates are presented in Table I. The rate of return of the human TR expressed in TRVb-1 cells is increased by a factor of 1.36 ± 0.05 (mean ± SD of four separate determinations). PMA affects the exocytosis of the hamster TR similarly, increasing the exocytic rate by a factor of about 1.50 ± 0.2 (mean ±SD of three separate determinations; Fig. 3, Table I). We have reported previously that the basal recycling rate of the hamster TR is faster than that of the human TR expressed in TRVb cells (Table I, McGraw et al., 1987). The increase in the rate of exocytosis correlates well with the increase in cell surface TR expression observed after PMA treatment (Figs. 1 and 2), suggesting that the increased surface expression is due to the effect on the recycling rate.

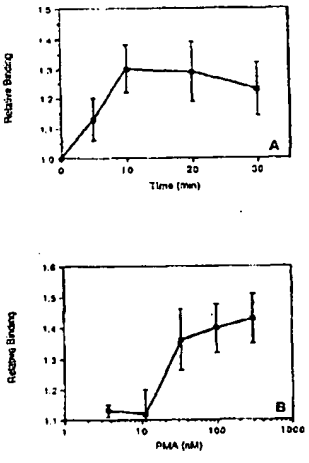

Figure 2. PMA effect on cell surface expression of the human TR in TRVb-1 cells. TRVb-1 is a CHO cell line, deficient in the expression of functional hamster TR, which expresses a cDNA clone of the human TR. *A* is a time course of the PMA effect on surface expression of the human TR. The cells were treated with 100 nM PMA in medium 1 at 37°C. The cells were cooled to 4°C and [^{125}I]Tf-binding was measured. The results shown are the mean ± SEM of three separate determinations of [^{125}I] Tf-binding relative to untreated cells. *B* is a dose–response curve of the PMA effect on cell surface TR expression. The cells were treated with PMA for 10 min. Surface [^{125}I]Tf-binding was determined as in *A*. The results presented are the means of three determinations ± SEM from a representive experiment.

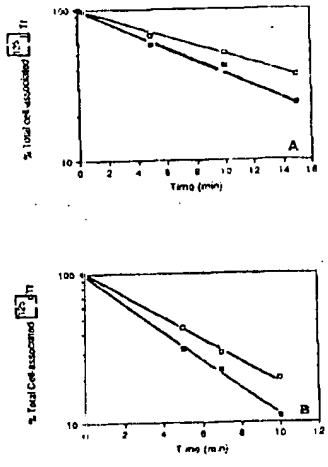

Figure 3. PMA effect on TR exocytosis in TRVb-1 and CHO cells. The rates of [^{125}I]apoTf release from TRVb-1 (*A*) and CHO cells (*B*) were calculated as described in Materials and Methods. The percent of total cell-associated [^{125}I]Tf remaining is plotted vs. time. The results presented are of representative experiments and are the means of triplicate determinations. *A* are TRVb-1 cells: (□) no treatment; (■) 100 nM PMA treatment. *B* are CHO cells: (□) no treatment; (■) 100 nM treatment.

Table I. The Effect of PMA Treatment on the Exocytic Rates of the Recycling Pathway of TR

Cell line	TR expressed	Exocytic rate of recycling min^{-1}	
		No PMA	*100 nM PMA*
CHO	Hamster TR	0.060 ± .006	0.090 ± .001
TRVb-1	Human TR (Ser-24)	0.032 ± .003	0.044 ± .002
TRVb-GlyA	Human TR (Gly-24)	0.042 ± .002	0.053 ± .004
TRVb-GlyB	Human TR (Gly-24)	0.040 ± .003	0.051 ± .001

The exocytic rates were determined by calculating the slope of a plot of the log of the loss of cell-associated [^{125}I]Tf vs. time. The values presented are the mean rates ±SEM of at least three separate experiments.

The effect of PMA on the internalization rate of Tf was examined using the steady-state procedure of Wiley and Cunningham (1982). In two separate experiments the rate of [^{125}I]Tf internalization in TRVb-1 cells was not significantly affected by PMA treatment. Together with the measured effect on exocytosis and the lack of a change in the size of the recycling receptor pool, these data show that the increased TR surface expression is a result of an increased rate of exocytosis.

TR Ser-24 Phosphorylation is not Required for Receptor Functioning

PMA-activated protein kinase C has been shown to phosphorylate the human TR on the cytoplasmic residue Ser-24 (Davis et al., 1986*b*). To investigate whether Ser-24 phosphorylation is required for the PMA induced redistribution of the human TR expressed in CHO cells, we constructed an in vitro mutagenized receptor in which Ser-24 has been replaced with the nonphosphorylatable amino acid, Gly. The mutated cDNA clone was transfected into TRVb cells and two independently derived cell lines were isolated: TRVb-Gly24A and TRVb-Gly24B.

The Gly substitution for Ser had no major effect on the behavior of the transfected receptor. Scatchard analysis of [^{125}I]Tf binding at 4°C to these cell lines demonstrated that the Gly substitution did not affect the affinity of the TR for Tf (Table II). The two transfected cell lines express different numbers of the human TR, although neither varies greatly from the 60,000 hamster TR expressed in CHO cells.

Both the patterns of internalization of fluorescein-labeled Tf and the immunofluorescent localization of the receptor with the anti-human TR receptor monoclonal antibody B3/25 demonstrate that the Gly-24 TR is processed by the cell in a fashion indistinguishable from that of the wild-type receptor at the level of resolution of light microscopy (not shown).

Table II. Dissociation Constants and Surface Expression of Cell Lines Transfected with Wild Type and Mutant Human TR

Cell line	K_d	Receptors/cell
	nM	
TRVb-1	1.5 ± 0.1	140,000 ± 12,000
TRVb-GlyA	2.4 ± 0.7	35,000 ± 10,000
TRVb-GlyB	1.4 ± 0.3	90,000 ± 10,000

The dissociation constants and surface receptor expression were calculated by Scatchard analysis of 4°C [^{125}I]Tf binding. The results represent the mean ±SD of at least three different experiments.

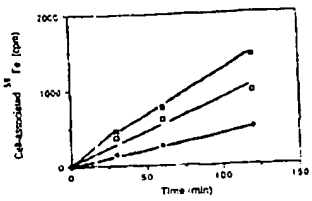

Figure 4. ^{59}Fe accumulation in TRVb-1, TRVb-GlyA, and TRVb-GlyB cells. TRVb-1 (■), TRVb-GlyA (♦), TRVb-GlyB (□), cells were incubated with 2 μg/ml Tf^{59}Fe$_2$ at 37°C. Cell-associated ^{59}Fe was determined by solubilizing the monolayers after washing. The data is presented as ^{59}Fe accumulation (cpm) per 1 ± 10^6 cells. Nonspecific ^{59}Fe accumulation was determined by incubation in the presence of a 500-fold excess of unlabeled Tf.

The exocytic rate of the recycling pathway is shown in Table I for the two cell lines containing the altered receptor. [^{125}I]Tf internalized by the two cell lines expressing the Gly-24 receptor is recycled and released from the cells with kinetics somewhat faster than that of the wild type receptor expressed in TRVb-1 cells (Table I). We do not know if the variation in basal exocytic rates in the cell lines shown in Table I reflects clonal variation among cell lines or if there is some receptor sequence effect.

The Gly-substituted receptor functions in delivery of iron to the cells. The mutant receptors accumulate ^{59}Fe from [^{59}Fe$_2$]Tf at a rate approximately proportional to the level of receptor expression (Fig. 4). Since the Gly substitution does not affect the behavior of the TR in any of the above respects, it can be concluded that Ser-24 phosphorylation is not required for the normal functioning of the TR.

Although Ser-24 phosphorylation is not required for the normal functioning of the receptor, it could be required for the PMA-induced redistribution. To investigate this possibility, the effect of 100 nM PMA on the exocytic rate of the Gly-

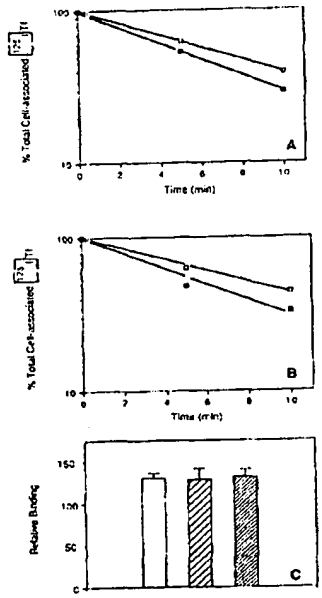

Figure 5. PMA effect on the exocytosis of Gly-24 substituted human TR. The rates of [^{125}I]apoTf release from TRVb-Gly24A and TRVb-Gly24A cells were calculated as described in Materials and Methods. The percent of total cell-associated [^{125}I]Tf remaining is plotted vs. time. The results presented are of representative experiments and are the means of triplicate determinations. *A* are TRVb-GlyA cells: (□) no treatment; (■) 100 nM PMA treatment. *B* are TRVb-GlyB cells: (□) no treatment; (■) 100 nM treatment. (*C*) PMA effect on TR surface expression measured by [^{125}I]Tf-binding at 4°C. The cells, □, TRVb-1; ▨, TRVb-Gly24A; ▩, TRVb-Gly24B; were incubated with 100 nM PMA for 10 min then cooled to 4°C and incubated with 2 μg/ml [^{125}I]Tf for 2 h. Binding after PMA treatment is presented relative to control binding. The results are the means of three experiments ± SD.

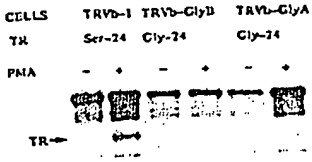

Figure 6. Immunoprecipitation of PMA-stimulated phosphorylation of the wild-type human TR from TRVb-1, and the Gly-24-substituted receptor from TRVb–Gly24A and TRVb–Gly24B cells. Cells were incubated with 1.0 mCi/ml [^{32}P]orthophosphate for 4 h. For PMA treatment 100 nM PMA was added for the final 30 min of the labeling incubation. The cells were lysed and an equal number of TCA precipitable counts from each lysate were used for the immunoprecipitation. The TR was precipitated with the human TR specific monoclonal antibody B3/25 after a previously described procedure (Lipsich et al., 1983). 50 μM Na_3VO_4 was present in the lysis and immunoprecipitation buffers to inhibit phosphatases. After boiling the immunoprecipitates in sample buffer, one-half (by volume) of the total immunoprecipitated protein from each lysate was loaded per lane of a 7.5% SDS–polyacrylamide gel under reducing conditions. To insure that all the TR was detected the beads were not extensively washed. This results in an increase in nonspecific binding to the beads. The nonspecific pattern was identical whether or not B3/25 was included in the immunoprecipitation.

mutated TR was examined. In both cell lines PMA treatment results in an increase in the rate of Tf release (Fig. 5; Table I). In TRVb–Gly24A there was a 1.32 ± .05 fold (mean ± SD) and in TRVb-Gly24B a 1.26 ± .02 fold (mean ± SD of four separate determinations) increase in the rate of Tf release. Furthermore, the surface TR expression, measured by [^{125}I]Tf binding at 4°C, is increased 30–40% by 100 nM PMA treatment (Fig. 5 *C*). These results demonstrate that Ser-24 phosphorylation is not required for the PMA-induced increase in the exocytic rate of the human TR expressed in TRVb cells.

The human TR is a disulphide linked homodimer of 96,000-D chains (Trowbridge et al., 1984). Since the receptors is a dimer it is possible that heterodimers between the transfected receptor and the endogenous receptor could form when the cDNA clone is expressed in a heterologous cell system. The TR-variant CHO cell line used in this study, TRVb cells, is devoid of functional endogenous TR. However, we have not characterized the mutation responsible for this phenotype. It is possible that TRVb cells express hamster TR which are mutated in the Tf-binding site. If this were the case, it would be possible to form heterodimers between the human TR monomer, introduced by transfection, and the mutant hamster receptor. In such a heterodimer the hamster cytoplasmic portion could complement the site-directed mutation in human receptor. Although we cannot directly rule out this possibility, we think that it is unlikely for several reasons. If heterodimers were formed, we would expect that the proportion of heterodimers in the transfected cell lines would be substantially different from one another due to the different amounts of transfected TR expressed in each cell line (35,000 and 90,000 TR per cell). However, in all measured respects the receptors behave identically in both Gly-24 transfectants. Therefore we do not believe that heterodimers are masking an effect of the Gly-24 substitution on the functioning of the TR.

To confirm that the Gly-24 substitution abolishes receptor phosphorylation, the TR was immunoprecipitated from TRVb-1, TRVb-Gly24A, and TRVb-Gly24B cell lines preincubated with [^{32}P]-orthophosphate and treated with 100 nM PMA for 15 min (Fig. 6). To account for any differences among the cell lines in the activity of protein kinase C or the incorporation of ^{32}P into ATP pools, an equal number of TCA precipitable counts from each lysate were used in the immunoprecipitation. Equal aliquots (by volume) of sample buffer containing total immunoprecipitated protein from each lysate were loaded per lane of the gel. PMA treatment stimulates phosphorylation of the wild type human TR expressed in TRVb-1 cells. As expected the Gly-24 substitution greatly reduced PMA-stimulated TR phosphorylation. In TRVb-Gly24A cells no phosphorylation of the TR is observed (Fig. 6). There is, however, a small amount of PMA-stimulated phosphorylation of the TR in TRVb-Gly24B cells (not visible in Fig. 6). This result suggests that there may be other minor site(s) of TR phosphorylation when expressed in TRVb cells. This minor phosphorylation is not a universal characteristic of expression of the human Gly-24 TR in TRVb cells since in cell line TRVb-Gly24A no phosphorylation is detected, even when the gels were over exposed to account for differences in TR number. This result confirms our conclusion that the phosphorylation of Ser-24 is not required for the PMA-induced TR redistribution.

Discussion

The surface expression of TR is regulated both pre- and posttranslationally. Receptor expression is decreased when cells are treated with Tf-alternative iron sources such as iron salts or hemin, and it is increased by chelating intracellular iron (Bridges and Cudkowicz, 1984; Ward et al., 1984; Rao et al., 1985). Iron-dependent regulation of TR expression is reflected by changes in mRNA concentration, suggesting that the alteration in receptor expression is regulated at the level of receptor synthesis (Rao et al., 1986). TR surface expression is also regulated posttranslationally. In this report we show that in CHO cells, the tumor promoting phorbol ester PMA causes an increase in the cell surface expression of the TR. PMA affects the steady-state distribution of TR in CHO cells by inducing an increase in the rate of exocytosis of the recycling receptor. PMA has been shown to have a similar effect on the TR in mouse tumor macrophage-like cells, J774 (Buys et al., 1984). The PMA effect is, however, cell line dependent. In K562 and HL60 cells PMA causes a decrease in surface expression by stimulating internalization of the TR (Klausner et al., 1984; May et al., 1984; May et al., 1985). Insulin and epidermal growth factor cause an increase in cell surface TR expression in fat cells and human fibroblasts (Davis et al., 1986*a*; Wiley and Kaplan, 1984). Recently, it has been shown that insulin increases the rate of recycling of the TR in adipocyte cells (Tanner and Lienhard, 1987). Thus, surface expression of the TR is under complex regulation both pre- and posttranslationally.

An attractive proposal for posttranslational regulation was that a modification of the TR resulted in the observed steady-state redistribution of intracellular and surface receptors. Since the protein kinase C activating phorbol ester PMA is capable of inducing a redistribution and the human TR is phosphorylated by protein kinase C, it was possible that TR phosphorylation was responsible for the observed effects. By characterizing the behavior of the human TR in which the major protein kinase C phosphorylation site, Ser-24, was

replaced with a non-phosphorylatable amino acid, Gly, we have shown that phosphorylation of Ser-24 is not required for the PMA induced redistribution. Furthermore, the Gly-24 TR behaved similarly to the wild type Ser-24 receptor in all aspects of endocytosis examined, suggesting that Ser-24 phosphorylation is not required for the functioning of the receptor. TRVb cells do not proliferate when the only Fe source is differic-Tf, presumably because of the lack of endogenous TR. The TRVb-Gly24 transfected cell lines do proliferate under these growth conditions, which indicates the complete biological functioning of the Gly-24 TR. Although our results demonstrate that Ser-24 phosphorylation is not required for the PMA effect on human TR expressed in CHO cells, it is still possible that phosphorylation of Ser-24 is involved in the growth factor-induced increase in TR surface expression or in the PMA-induced TR internalization in K562 and HL60 cells.

Our results suggest that phosphorylation of protein(s) other than the TR is responsible for regulating the trafficking of this membrane protein. The increased exocytic rate could result from a general increase in the rate of fusion of the recycling vesicles with the plasma membrane or from an increase in the rate of movement of the intracellular vesicles to the site of fusion with the cell membrane. Identifying the intracellular location of the action of PMA treatment requires a detailed morphological study of the exocytic portion of the TR recycling pathway.

Our results raise the question as to whether the PMA effect is specific for the TR (or a subclass of membrane proteins of which the TR is a member) or if the TR redistribution reflects a general perturbation of cellular membrane traffic. Further characterization of a number of recycling membrane proteins in one cell system will be required to answer this question.

A report on the requirement of TR phosphorylation for endocytosis of the receptor has recently appeared (Rothenberger et al., 1987). Our result that Ser-24 phosphorylation is not required for basal functioning of the TR is in agreement with their results. In their study they have expressed the human TR in mouse LTK$^-$ cells, which do not respond to PMA treatment. They were, therefore, unable to investigate the role of TR phosphorylation in response to PMA. In a more recent study it has been shown that PMA up-regulates cell surface TR expression in mouse 3T3 cells and that this up-regulation is independent of TR phosphorylation (Zerial et al., 1987). These results are also consistent with our results. In our study we have taken advantage of a heterologous system lacking endogenous TR. This has allowed us to characterize the up-regulation of TR in detail using the native ligand.

This heterologous system is ideal for future morphological and biochemical studies of the exocytic portion of the TR recycling pathway directed at identifying the site of action of PMA on steady-state receptor distribution.

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Note added in proof: Similar results to those reported here have recently been published by R. J. Davis and H. Meisner 1987. *J. Biol. Chem.* 262: 16041-16047.

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Requirement for negative charge on "activation loop" of protein kinase C.

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Increasing evidence has implicated a post-translational phosphorylation in the production of a catalytically competent protein kinase C. Here we present structural and biochemical evidence that Thr500 of protein kinase C-beta II is the residue phosphorylated by another kinase. Modeling studies indicate that this residue is part of a "lip" structure at the entrance of the catalytic site; phosphorylation on this lip, or "activation loop," is central to the regulation of three kinases whose structures have been elucidated (Taylor, S. S., and Radzio-Andzelm, E. (1994) Structure 2, 345-355). Biochemical data reveal that mutation of Thr500 to an acidic residue (Glu) results in expression of catalytically active protein kinase C in COS cells. In contrast, mutation of this residue to a neutral, non-phosphorylatable residue (Val) results in expression of inactive enzyme. Thus, negative charge at position 500 is required for catalytically competent protein kinase C-beta II. These data suggest that signal processing by protein kinase C cannot occur until the enzyme is first phosphorylated by a protein kinase C kinase.

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A phosphorylation site in the ftz homeodomain is required for activity.

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The Drosophila homeodomain-containing protein Fushi tarazu (Ftz) is expressed sequentially in the embryo, first in alternate segments, then in specific neuroblasts and neurons in the central nervous system, and finally in parts of the gut. During these different developmental stages, the protein is heavily phosphorylated on different subsets of Ser and Thr residues. This stage-specific phosphorylation suggests possible roles for signal transduction pathways in directing tissue-specific Ftz activities. Here we show that one of the Ftz phosphorylation sites, T263 in the N-terminus of the Ftz homeodomain, is phosphorylated in vitro by Drosophila embryo extracts and protein kinase A. In the embryo, mutagenesis of this site to the non-phosphorylatable residue Ala resulted in loss of ftz-dependent segments. Conversely, substitution of T263 with Asp, which is also non-phosphorylatable, but which successfully mimics phosphorylated residues in a number of proteins, rescued the mutant phenotype. This suggests that T263 is in the phosphorylated state when functioning normally in vivo. We also demonstrate that the T263 substitutions of Ala and Asp do not affect Ftz DNA-binding activity in vitro, nor do they affect stability or transcriptional activity in transfected S2 cells. This suggests that T263 phosphorylation is most likely required for a homeodomain-mediated interaction with an embryonically expressed protein.

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